University of Alberta

Liposomal Delivery of Intracellular Trehalose

by

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***This achievement is dedicated to my parents,
Lepa and Slavko Lecak, who are my inspiration.
Thank you for all your love and support throughout my life.
Volim vas zauvijek.***

ABSTRACT

Cryopreservation of RBCs for transfusion is one of the most important applications of cryobiology research. The major advances in red blood cell cryopreservation occurred in the 1950s and 1960s, with the development of clinically-safe techniques for red blood cell freezing, storage, thawing and post-thaw processing. The current red blood cell cryopreservation is still based on these traditional methods using glycerol as a cryoprotectant, although they remain labour-intensive and expensive procedures that result in red blood cells with shorter post-transfusion in vivo survival. The relatively low concentrations of non-toxic disaccharide trehalose have been reported to act as an effective cryoprotective agent, when available on both sides of cell membrane. However, red blood cell membranes are impermeable to trehalose.

This thesis tested the hypothesis that liposomal delivery can be used as a permeabilization strategy for the intracellular accumulation of trehalose with minimal injury to red blood cell membranes, providing a novel approach for red blood cell cryopreservation. The main objective of this thesis was to increase our understanding of the effects of liposome-red blood cell interaction on red blood cell response to freezing, in order to guide a liposome-based approach to improved red blood cell cryopreservation for transfusion medicine applications.

The work presented in this thesis has provided a further understanding of subtle red blood cell membrane injury that occurs during traditional clinical red blood cell cryopreservation. Furthermore, this thesis described the first account

of using liposomes as membrane permeabilization strategy for intracellular delivery of trehalose. In the process of investigating liposomes as intracellular trehalose delivery vesicles, this thesis has contributed comprehensive analysis of interactions of trehalose-containing liposomes and human red blood cells, including modes of interactions and the effects of various experimental parameters on liposome-red blood cell interaction, trehalose loading and red blood cell membrane quality. Finally, this thesis has advanced the knowledge base of cryobiology by providing novel evidence that liposomes can be used to exert a cryoprotective response in human red blood cells, which may change how cryoprotection is viewed, leading the research into a whole new class of cryoprotective agents.

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Table of Contents

Chapter 1:

Introduction **1**
1.1. Cryobiology 2
  1.1.1. Cell Responses to Freezing and Theories of Cryoinjury 3
  1.1.2. Cryoprotectants 5
1.2. Trehalose 7
  1.2.1. Properties 7
  1.2.2. Mechanisms of Biostabilizing Action 9
  1.2.3. Applications in the Biopreservation Field 10
1.3. RBC Cryopreservation 13
  1.3.1. Why Preserve RBCs? 13
  1.3.2. Clinical RBC Cryopreservation 15
  1.3.3. Shortcomings of Traditionally Cryopreserved RBCs 18
1.4. Thesis Approach 20
1.5. Hypothesis and Thesis Objectives 22
1.6. References 24

Chapter 2:

The Effects of Cryopreservation on Erythrocyte Microvesiculation, Phosphatidylserine Externalization and CD47 Expression **33**
2.1. Introduction 34
2.2. Materials and Methods 38
  2.2.1. RBC Processing 39
  2.2.2. RBC Quality Assessment 40
    2.2.2.1. Conventional RBC Quality Assessment 40
    2.2.2.2. RBC Quality Assessment Using Flow Cyotometry 45
    2.2.2.3. Additional Post-thaw RBC Quality Assessment 46
  2.2.3. Statistical Analysis 46
2.3. Results 47
2.4 Discussion 51

2.5 Conclusion 59
2.6 References 74

Chapter 3:

Synthesis and Characterization of Trehalose-containing Liposomes for Applications in Biopreservation 80
3.1. Introduction 81
3.2. Materials and Methods 83
  3.2.1. Liposome Synthesis 83
  3.2.2. Liposome Characterization 85
    3.2.2.1. Liposome Phosphate Content 85
    3.2.2.2. Liposome Size 87
    3.2.2.3. Liposome Morphology 88
    3.2.2.4. Liposome Number 89
    3.2.2.5. Intra-liposomal Trehalose Content 89
3.3. Results 92
3.4 Discussion 95
3.5 Conclusion 102
3.6 References 114

Chapter 4:

Investigating Interactions of Trehalose-containing Liposomes with Human Red Blood Cells 121
4.1. Introduction 121
4.2. Materials and Methods 125
  4.2.1. Synthesis and Characterization of Fluorescently-labelled Trehalose-containing Liposomes 125
  4.2.2. Liposome Incubation with RBCs 127
  4.2.3. Density Gradient Separation 128
  4.2.4. Monitoring Liposome Interactions with RBCs 128
4.3. Results 130

4.4 Discussion ... 133
4.5 Conclusion ... 140
4.6 References ... 152

Chapter 5:
Greasing Fusion Machinery: Experimental Parameters Affecting Liposome-RBC Interactions and RBC Membrane Quality ... 158
5.1. Introduction ... 159
5.2. Materials and Methods ... 163
  5.2.1. The Effects of Incubation Conditions on Liposome-RBC Fluorescence Transfer ... 163
  5.2.2. The Effect of Liposome Aqueous Core Composition on Intracellular Trehalose Delivery ... 164
  5.2.3. The Effect of Liposome Membrane Composition on Liposomal Trehalose Delivery ... 165
  5.2.4. The Effects of Liposomes on RBCs Membrane Quality ... 166
5.3. Results ... 167
  5.3.1. The Effects of Incubation Conditions on Liposome-RBC Fluorescence Transfer ... 167
  5.3.2. The Effects of Liposome Composition on Liposome Trehalose Delivery ... 169
  5.3.3. The Effects of Incubation Conditions on Liposome Trehalose Delivery ... 170
  5.3.4. The Effects of Liposomes on RBCs Membrane Quality ... 170
5.4 Discussion ... 171
5.5 Conclusion ... 177
5.6 References ... 189

Chapter 6:

Effects of Trehalose-loaded Liposomes on RBC Response to Freezing and Post-thaw Membrane Quality 194
6.1. Introduction 195
6.2. Materials and Methods 199
  6.2.1. Liposomes, Lipids and RBCs 199
  6.2.2. Freezing Experiments 201
  6.2.3. RBC Post-thaw Quality Assessment 202
6.3. Results 204
  6.3.1. The Effects of Cooling Rate and Freezing Solutions on I-RBC Response to Freezing 204
  6.3.2. The Effects of Liposome Treatment and Charge on RBC Response to Freezing 206
  6.3.3. The Effects of Freezing on I-RBC Membrane Quality 206
  6.3.4. On the Mechanism of Liposome Cryoprotection 207
6.4 Discussion 208
6.5 Conclusion 215
6.6 References 231

Chapter 7:

General Discussion and Conclusions 235
7.1. Review of Thesis Objectives 236
7.2. Contributions to Science and Future Directions 241
7.3. References 244

List of Tables

TABLE	TITLE	PAGE
Table 2.1	RBC quality measures in 2-3 d pre-freeze storage group. There was no statistically significant difference between the pre-freeze RBC quality results and the hypothermic controls for any of the assays performed	63
Table 2.2	RBC quality measures in 13-14 d pre-freeze storage group. There was no statistically significant difference between the pre-freeze RBC quality results and the hypothermic controls for any of the assays performed.	64
Table 3.1	Hydrating and external buffers used in liposome synthesis	105
Table 4.1	Physical characteristics of fluorescently-labelled and control liposomes synthesized in the study	141
Table 4.2	Concentration of trehalose in red blood cells following interaction with liposomes. Delivery of intraliposomal content was confirmed by intracellular trehalose measurements (n=3).	142
Table 6.1	Abbreviations used to describe different liposome preparations and treatments of RBCs	217

List of Figures

FIGURE	TITLE	PAGE
Figure 2.1	The experimental design of this study	61
Figure 2.2	The number counted of each cell type is multiplied by the shape factor. The morphological index for each RBC sample is then calculated by adding these products. The closer the morphological index is to 100, the closer the morphology of the sample is to the morphology of normal RBCs.	62
Figure 2.3	A forward scatter (FSC) versus side scatter (SSC) plot with logarithmic scales was used to set a region around the RBC population (R1) and microvesicle population (R2). RBC- and microparticle-associated fluorescence intensity for annexin V and CD47 markers was then measured.	65
Figure 2.4	PS exposure of cryopreserved RBCs. After 24 h of post-thaw storage a significant increase in PS exposure was seen in the 13-14 d stored units as compared to the 2-3 d stored units (*p=0.016). In addition, following 24 h of post-thaw storage, PS exposure was significantly higher compared to pre-freeze levels (*p=0.021).	68
Figure 2.5	CD47 expression of cryopreserved RBCs.In both test groups, CD47 expression did not significantly change after cryopreservation or following the 24-h post-thaw period.	69
Figure 2.6	Microvesiculation of cryopreserved RBCs. While pre-freeze and post-thaw hypothermic storage resulted in microvesicle accumulation, the freezing process itself did not contribute to microvesiculation. Prolonged pre-freeze storage resulted in significant accumulation of microvesicles after 24 h of post-thaw hypothermic storage (p=0.017).	70
Figure 2.7	PS expression of RBC microparticles. After 24 h of post-thaw storage, a significant increase in PS fluorescence intensity per microparticle compared to pre-freeze values was seen in the both 2-3 d and 13-14 d stored units (*p<0.001).	71

FIGURE	TITLE	PAGE
Figure 2.8	CD47 expression of RBC microparticles. There was no statistically significant difference in CD47 fluorescence intensity per microparticle between the test groups or between time points.	72
Figure 2.9	The average length of time packed RBC units are stored hypothermically prior to cryopreservation in CBS Edmonton Centre between 1993 and 2002 is substantially less than that permitted by existing standards (7.3 ± 2.0 d, range 1-40 d), but has continuously increased over the past 10 years (1993-1997 average 5.9 ± 1.8 d; 1997-2002 average 8.6 ± 0.9 d).	73
Figure 3.1	Experimental steps for synthesis and characterization of trehalose-containing liposomes.	104
Figure 3.2	Previously published data relating the number of phospholipid molecules in a single vesicle as a function of vesicle radius was fitted to a 10-degree polynomial equation to determine the number of phospholipid molecules in a liposome vesicle of known size	106
Figure 3.3	Spectrophotometric assay of the phosphate content was used to determine the total lipid concentration of the different liposome preparations, shown as triangles.	107
Figure 3.4	Repeated extrusion resulted in a unimodal, normal liposomal distribution, suggesting homogeneity of synthesized trehalose-containing liposomes that is independent of liposome concentration inside liposomes.	108
Figure 3.5	Standard latex beads (400 nm, top left picture) and synthesized liposomes were visualized by TEM.	109
Figure 3.6	Sensitivity and linearity of spectrophotometric (Fig. A) and HPLC-ELSD trehalose measurement methods (Fig B).	110
Figure 3.7	The spectrophotometrically-measured trehalose concentration of the 1 mM lipid liposomal lysates	111
Figure 3.8	The spectrophotometrically-measured trehalose concentration of the 1 mM lipid liposomal lysates were confirmed with the HPLC measurements.	112

FIGURE	TITLE	PAGE
Figure 3.9	HPLC was performed on the liposomal supernatants prior to lysis in order to assess whether trehalose was only present in the liposomal aqueous core. Fructose and lactose were used as internal standards.	113
Figure 4.1	Schematic of the possible modes of interactions between trehalose-containing liposomes and mammalian cells. As mature human RBCs are unable to endocytose, there are three possible outcomes: no interaction, liposome adsorbtion to RBC membranes, and trehalose delivery through liposome fusion.	143
Figure 4.2	R18 trehalose-containing liposome interacting with RBCs. Red fluorescence (FL2-H) as a function of forward side scatter (FSC-H) (Figure A) depicts a highly fluorescent RBC population when mixed with the quenched R18 liposomes. The fluorescence histogram (Figure B) illustrates dequenching of R18 marker in RBC membrane upon transfer from liposome population.	144
Figure 4.3	Effect of density gradient separation on R18 liposome interaction with RBC. Density gradient separation removed the majority of R18 liposomes (Figure B) but did not result in marked reduction of RBC mean fluorescence intensity (Figure A).	145
Figure 4.4	Fluorescent microscopy image of dequenching of R18 marker in RBC membrane after interaction with R18 liposomes.	146
Figure 4.5	Flow cytometric analysis of 5(6)-CF liposomes (Figure A) and control RBCs (Figure B) with corresponding fluorescence histograms (Figure C) before incubation and interaction. Green fluorescence (FL1-H) is plotted as a function of forward side scatter (FSC-H) (Figure A and B).	147
Figure 4.6	Flow cytometric analysis of 5(6)-CF liposomes and RBCs after incubation (Figure A and B). The fluorescence histogram (Figure B) illustrates transfer of the 5(6)-CF to the RBC membrane resulting in fluorescent RBC.	148

FIGURE	TITLE	PAGE
Figure 4.7	Effect of density gradient separation (Percoll) on the interaction of trehalose-containing liposomes with human RBC. Density gradient separation was effective at removing the majority of the 5(6)-CF liposomes from solution (Figure A), however it also resulted in a decrease in the transfer of 5(6)-CF to RBC (Figure B).	149
Figure 4.8	Fluorescent microscopy image of RBCs after interacting with 5(6)-CF liposomes.	150
Figure 4.9	Flow cytometry and confocal microscopy images show: control RBC (Figure A), transfer of hydrophobic fluorescent marker R18 (Figure B), hydrophilic marker 5(6)-CF (Figure C) and both markers (Figures D and E) following incubation with 5(6)-CF-R18 liposomes to RBCs.	151
Figure 5.1	The effect of liposome concentration on fluorescence transfer from 5(6)-CF labelled liposomes to RBCs before Percoll density gradient separation (Figure A) and after Percoll density gradient separation (Figure B).	179
Figure 5.2	The effect of incubation time (Figure A) and incubation temperature (Figure B) on fluorescence transfer from 5(6)-CF labelled liposomes to RBCs.	180
Figure 5.3	The effect of liposome aqueous core composition and liposome concentration on intracellular trehalose loading. Concentrations of the uncharged liposomes (2, 4 and 6 mM lipid) containing 200, 300 and 400 mM trehalose in their aqueous core were incubated with human RBCs for 2 h at 37 °C.	181
Figure 5.4	The effects of liposome concentration and charge on intracellular trehalose loading. Concentrations of the charged and uncharged liposomes (2, 4 and 6 mM lipid) were incubated with human RBCs for 4 h at 37 °C.	182
Figure 5.5	The effects of incubation time and liposome charge on intracellular trehalose loading. Charged and uncharged liposomes (4 mM lipid) were incubated with human RBCs for 1, 4 and 24 h at 37 °C.	183

FIGURE	TITLE	PAGE
Figure 5.6	The effect of extracellular solution on intracellular trehalose loading after 4 h incubation with 4 mM liposomes at 37 °C.	184
Figure 5.7	The effect of liposome treatment and subsequent hypothermic storage on percent hemolysis	185
Figure 5.8	The effect of liposome treatment on RBC morphology, PS exposure and CD47 expression	186
Figure 5.9	To examine the effect of incubation time on PS exposure, 4 mM of negatively charged liposomes were incubated with RBCs at 37 °C for 2, 4, 24 and 48 h (Figure A) while the effect of incubation temperature on PS exposure was investigated by incubating 4 mM of negatively charged liposomes with RBCs at 4°C, RT and 37 °C for 24 h (Figure B).	187
Figure 5.10	The effect of liposome concentration and charge on RBC recovery	188
Figure 6.1	Cell survival as a function of cooling rate is illustrated, with the resulting injury occurring at slow cooling rates (solution effects injury) and at rapid cooling rates (intracellular ice formation).	218
Figure 6.2	Cooling rate profiles: Figure A illustrates slow cooling rate, Figure B medium and Figure C fast cooling rate. The average cooling rate was calculated from temperature and time measurements as samples were cooled from -5 °C (after the dissipation of the latent heat of fusion) to -40 °C, as demonstrated by the blue box.	219
Figure 6.3	The effects of different cooling rates and freezing solutions on post-thaw recovery of liposome-treated and control RBCs.	221
Figure 6.4	The effects of different cooling rates and freezing solutions on post-thaw recovery of liposome-treated and control RBCs.	222
Figure 6.5	Pre-freeze (A) and post-thaw (B) morphology assessment of I-RBCs.	223

FIGURE	TITLE	PAGE
Figure 6.6	Pre-freeze (A) and post-thaw (B) morphology assessment of I-RBCs.	224
Figure 6.7	Flow cytometry analysis of liposome-treated RBCs, shown in the gate R1, pre-freeze (Figure A) and post-thaw (Figure B).	225
Figure 6.8	Flow cytometry analysis of control RBCs, shown in the gate R1, pre-freeze (Figure A) and post-thaw (Figure B).	226
Figure 6.9	Pre-freeze and post-thaw flow cytometry analysis of PS exposure and CD47 expression of liposome-treated and control RBCs.	227
Figure 6.10	Pre-freeze and post-thaw flow cytometry analysis of PS exposure and CD47 expression of liposome-treated and control RBCs.	228
Figure 6.11	Phospholipid delivery during liposome-RBC membrane fusion plays an important role in improving RBC survival after freezing. Please refer to Table 6.1 for the abbreviations used to describe different treatments of RBCs.	229
Figure 6.12	This graph illustrates cell survival as a function of cooling rate of liposome-treated (in green) and control RBCs (in black) frozen in NaCl solution with slow, intermediate and fast different cooling rates, and compares our results to previously published survival curves of RBCs frozen without CPA, (in black) RBCs frozen with a high concentration of permeating CPA glycerol (in red), and RBC frozen in a 10 % sucrose solution, a nonpermeating CPA (in blue).	230

List of Abbreviations

AABB	American Association of Blood Banks
ANOVA	analysis of variance
ATP	adenosine triphosphate
Ba^{2+}	barium divalent cation
Ca^{2+}	calcium divalent cation
$CaCl_2$	calcium chloride
CBS	Canadian Blood Services
CCD	charge-coupled device
CPA	cryoprotective agent
CPD	citrate-phosphate-dextrose solution
c-RBC	control RBCs
Cy5	cyanine-5
DPPC	dipalmitoylphosphatidylcholine
ELSD	evaporative light scattering detector
FACS	fluorescent activated cytometry
FSC	forward scatter
GC-MS	gas chromatography-mass spectroscopy
HBS	HEPES buffered saline
Hct	hematocrit
HES	hydroxyethyl starch
HiCN	cyanmethemoglobin
HPLC	high performance liquid chromatography

IgG	immunoglobulin G
l-RBC	liposome-treated RBCs
LUV	large unilamellar vesicle
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
Mg^{2+}	magnesium divalent cation
MLV	multilamellar vesicle
NA	not available
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	monosodium phosphate
NBRL	Naval Blood Research Laboratory
NEM	N-ethylmaleimide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PS	phosphatidylserine
PVP	polyvinylpyrrolidine
R18	rhodamine B chloride
RBC	red blood cell
RT	room temperature (21 °C)

SAGM	saline-adenine-glucose-mannitol
SCC	side scatter
SD	standard deviation
SM	sphingomyelin
SRA	specific research aim
SSE	sum of squares due to error
SUV	small unilamellar vesicle
TEM	transmission electron microscopy
T_g	glass transition temperature
T_m	gel to liquid-crystalline phase transition temperature
2,3-DPG	2,3-diphosphoglycerate
5(6)-CF	5(6)-carboxyfluorescein

Chapter 1*

Introduction

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1.1. Cryobiology

Cryobiology is a multidisciplinary science that studies the biological and physical behaviour of cellular systems at low temperatures. Although the term 'cryobiology' was originally coined in the 1950s using the Greek word *kruos* (meaning cold) to derive the prefix "cryo", early publications in low temperature biology date back to the 17th century.[1] In 1663, Robert Boyle published a famous book "New Experiments and Observations Touching Cold" which documented freezing experiments on living organisms.[2] Attracted by the prospects of suspended animation, numerous scientists in the 18th and 19th centuries furthered cryobiology research. For example, in the 1860s Pouchet froze entire animals (frogs, eels, cats) to study the freezing point of animal blood.[3] However, one book is often credited with the birth of cryobiology science. The book "Life and Death at Low Temperatures", written by Luyet in 1940, described and proposed mechanisms by which ice damages cells and tissues, which became the essence of cryobiology research.[4] Also considered a father of "transfusion cryobiology" for his work on RBC preservation, Luyet was honoured as the first president of the Society for Cryobiology for his 40 years of scientific contribution to the field.[3; 4]

As an applied science, cryobiology is primarily concerned with low temperature preservation, or cryopreservation. Metabolic and biochemical reactions essentially cease at ultra low temperatures. Therefore, cells can be held in biological stasis. Consequently, cellular viability and function can be preserved outside the native environment for prolonged periods of time, which offers enormous practical benefits. Cryopreservation principles are applied in numerous disciplines, including medicine, biotechnology, agriculture, forestry, microbiology, pharmacology, and food sciences. Examples of medical applications are cryopreservation of RBCs for transfusion, bone marrow hematopoietic progenitor cells and pancreatic islets for transplantation, and gametes for assisted reproduction. In addition to preserving cells, cryobiology can be used to destroy them. For example, cryosurgery can be used as a minimally invasive approach for destruction of malignant tissue. Advances in cryopreservation have been made and will continue to develop with advances in the understanding of cell responses to freezing and multiple elements responsible for low-temperature injury.

1.1.1. Cell Responses to Freezing and Theories of Cryoinjury

When a cell solution is subjected to subzero temperatures, ice will initially form in the extracellular medium, as the cell cytoplasm does not contain effective ice nucleators and the cell membrane is an effective barrier to ice growth.[5] Therefore, cells generally remain unfrozen, and thus supercooled. As ice forms outside of the cells, pure water is "precipitated" in the form of fixed lattice of the ice crystal. Ice has a limited ability to incorporate solutes, so the solutes become increasingly concentrated in the remaining unfrozen liquid. This progressive increase in solute concentration translates into higher osmolality in the extracellular solution compared to the intracellular solution, resulting in an osmotic pressure gradient. According to Mazur's famous "two-factor hypothesis", further events, including the manner in which cells respond and the source of their cryoinjury, will largely depend on the rate at which they are cooled.[6; 7]

During slow cooling, the cell is able to maintain osmotic equilibrium with the extracellular solution by becoming increasingly dehydrated. In these conditions, cryoinjury is related to these "solution effects." The exact mechanism of cryoinjury due to solution effect is unknown and many theories have been proposed. For example, Mazur and colleagues hypothesized that this injury is due to the effect of cell exposure to concentrated extra- and intracellular solutions at high sub-zero temperatures.[6; 7] Meryman proposed the "minimum cell volume" hypothesis, suggesting that solution effect injury results from the decrease in intracellular volume beyond a critical minimum.[8] Stepenkus *et al.* suggested that the surface area of the cell is reduced as the plasma membrane components are lost due to the stress of slow cooling cryoinjury.[9]

The second half of Mazur's "two-factor hypothesis" deals with cryoinjury related to high cooling rates. During rapid cooling, the formation of ice in the extracellular solution occurs too quickly for the cell to osmotically respond by losing water. Therefore, the cell cytoplasm becomes increasingly supercooled until it freezes. There has been significant evidence relating rapid cool cryoinjury to intracellular ice formation and there are several hypotheses attempting to explain the mechanism by which extracellular ice interacts with the plasma membrane to initiate intracellular ice formation.[6; 7; 10; 11]

In addition to cooling injury, cryoinjury is also associated with the warming process. For example, ice recrystallization, or the tendency of small intracellular ice crystals to grow into larger crystals upon slow warming, can significantly damage cells.[1; 8; 12; 13] Whether a given cooling / warming rate is too high or low for a given cell type depends on the water permeability of that cell's membrane.[7; 8; 13; 14] Understanding the mechanisms and causes of damage during cryopreservation supports the development of new cryopreservation protocols.

1.1.2. Cryoprotectants

Cryoinjury associated with cooling and warming can be minimized by adding cryoprotective agents (CPAs) to a cell suspension. The serendipitous discovery by Polge, Smith and Parkes in 1948 that spermatozoa could be frozen without loss of motility if glycerol was included in the suspending medium initiated an enormous amount of empirical research on different CPAs.[15] CPAs are traditionally separated into two broad classes, based on their ability to diffuse across cell membranes: penetrating CPAs are able to move across cell membranes, whereas non-penetrating CPAs cannot. Penetrating CPAs, such as glycerol, dimethlysulfoxide and ethylene glycol are associated with protection against slow cooling injury. They act colligatively to depress the freezing point of a solution, reducing the amount of ice formed at any given temperature.[16; 17] Therefore, the solute concentration inside and outside the cell is reduced, which minimizes osmotic stress and excessive cell volume reduction.[16; 17]

However, these CPAs can be also damaging to cells. As the plasma membrane is more permeable to water than to CPAs, high concentrations of permeating CPAs can result in detrimental cell volume excursions.[16; 17] In addition, high concentrations of common permeating CPAs are cytotoxic.[16; 17]

Nonpermeating CPAs are generally larger molecules such as sugars, starches and polymers. The mechanism(s) of action of these molecules has not been as well described as those for permeating CPAs. It is thought that nonpermeating CPAs act by increasing the osmolality of the extracellular solution, so that cells dehydrate at high subzero temperatures. Therefore, the amount of intracellular water is reduced; cell cytoplasm becomes less supercooled, which is thought to decrease the incidence of intracellular ice formation during rapid cooling.[16; 17] In addition, it has been suggested that nonpermeating CPAs also act to stabilize the plasma membrane structures.[5; 16; 17; 18]

In addition to permeating and nonpermeating CPAs, nonpermeating intracellular CPAs are recently attracting a lot of attention in biopreservation research. The idea of intracellular CPAs comes from nature. In nature, a variety of organisms, including frogs, nematodes, tardigrades, insects, bacteria, yeast, and plants accumulate intracellular stabilizing molecules, such as sugars, glycols and proteins, to cope with extreme environmental conditions leading to freezing and desiccation.[1; 19; 20; 21; 22] Many studies on these adaptive strategies focused on the ability of trehalose to confer protection against desiccation, which led to further investigations on the use of trehalose as a novel CPA.

1.2. Trehalose

1.2.1. Properties

Trehalose is a disaccharide composed of two D-glucose molecules bound by an alpha-alpha linkage (α-D-glucopyranosyl-α-D-glucopyranoside).[23] It was first isolated in 1832 by Wiggers, who was studying ergot of rye when he noticed that the solution contained slightly sweet sugar crystals that were highly soluble in water and resistant to hydrolysis.[23] Berthelot coined the word trehalose from the "*trehala manna*", the protective cacoon shell that is secreted by several insects in the North Iraqian dessert and contains up to 30 % of this sugar.[24] He described local tribes gathering this product from leaves on bushes, and using it as a sweetening agent.[24] Since then, trehalose has been show to be widespread in nature, occurring in fungi, bacteria, plants, invertebrate and vertebrate organisms.[23; 25]

Trehalose is a non-reducing disaccharide. Since the reducing glucosyl carbon end is connected with the other by an alpha-alpha linkage, trehalose has no reducing power.[23; 25; 26] In addition, the alpha-alpha linkage is very stable, so trehalose is not easily hydrolyzed or enzymatically cleaved.[25; 27] Numerous additional properties make trehalose one of the most stable sugars. It has a low hydroscopic profile - the water content of trehalose dehydrate remains stable up to a relative humidity of 92 %.[25] It also has high thermostability and a wide pH stability range. When trehalose solutions with pH ranging from 3.5 to 10 were heated at 100 °C for 24 h, no degradation of trehalose was observed.[27] Trehalose exhibits a rich phase diagram, with various crystalline states, including dehydrate, anhydrous, hydrated amorphous and glass anhydrous.[28] The glass transition temperature (T_g) of the amorphous phase is the highest of all disaccharides, which is another important biostabilizing property.[29] The stability of trehalose in the glassy state is enhanced by its ability to regulate water content by locally converting into crystalline dehydrate upon adsorption of water vapour, thus sparing the remaining trehalose molecules from contact with water.[26]

Another attractive property of trehalose is its safety. Unlike many other cryoprotectants, trehalose is non-toxic.[25] It is currently approved for human use as a food additive in Canada, USA, Europe and Japan.[25] A Japanese taste panel has judged trehalose to be about 45 % as sweet as sucrose, with the perceived sweetness increasing about three times faster relative to sucrose.[30] Metabolism of trehalose is essentially identical to all other disaccharides: trehalose is enzymatically hydrolyzed into two glucose molecules, which are then absorbed and processed by normal biochemical mechanisms.[25] Therefore, in addition to biopreservation applications, trehalose's physical and chemical properties make it attractive in the food, heath, beauty and pharmaceutical industries.[25]

Trehalose functions are species dependent. For example, it is an energy source for germination of spores,[27] a structural element of mycobacteria,[31] and a metabolic intermediate providing energy for flight in insects.[32] However, the most fascinating function of trehalose is protection of cell structural integrity during freezing and desiccation.[23; 25; 33; 34; 35; 36; 37]

1.2.2. Mechanisms of Biostabilizing Action

Although trehalose is the most actively studied nonpermeating intracellular stabilizing agent, the exact mechanism(s) by which trehalose protects cells from cryo- and lyoinjury is still unknown. Studies have focused on the interacation of trehalose with plasma membrane[18; 38] and cell proteins,[39; 40; 41] formation of a protective glass matrix,[28; 37; 42] the effects on the solution properties of the cell cytoplasm,[27; 43; 44] and osmotic cell responses.[45; 46]

For example, Crowe *et al.* proposed a "water replacement hypothesis", suggesting that the unique orientation of the hydroxyl groups on the trehalose molecule make it an effective replacement of water molecules, so that proper folding of proteins during dehydration and dry storage can be maintained.[38] In addition, trehalose hydroxyl groups can bind to phospholipid headgroups of the lipid bilayer, depressing the membrane phase transition temperature (T_m) and consequently preventing cytoplasmic leakage during rehydration.[38]

Another prominent theory is the "vitrification hypothesis". Vitrification is the process of converting a material into a glass-like amorphous state. Low molecular mobility in this vitrified state results in decreased rates of deleterious biochemical reactions, so that biomolecules are protected from cryo- and lyo-injury.[29; 42] As the T_g of trehalose is highest for all disaccharides, trehalose suspensions may remain in the glassy state at ambient temperatures, which has been correlated with enhanced stability.[21; 47; 48]

The next theory on the mechanism of trehalose protection focuses on cell volume responses. The presence of intracellular trehalose may confer protection during freezing and drying by buffering damaging cell volume changes, especially during freezing and thawing, so that damage to both membranes and intracellular structures is minimized.[49; 50] Furthermore, as trehalose is a non-reducing sugar, the resistance to nonenzymatic browning has also been described as one of trehalose's biostabilizing properties.[23; 25; 26] Nonenzymatic browning reaction between reducing sugars and proteins has been correlated to cell injury, so trehalose alpha-alpha glucosyl carbon linkage and resistance to hydrolysis is beneficial to biostabilizaton applications.[25; 26]

Due to these multiple theories, Crowe *et al.* have pointed out that over the past two decades a myth has developed about the "special" properties of trehalose for stabilizing biomaterials.[26] They pointed out that under ideal conditions for storage, trehalose has few special properties.[26] However, under suboptimal conditions, such as high storage temperatures and high relative humidities, using trehalose as a biostabilizing agent has distinct advantages.[26] These multiple theories also suggest that our understanding of the biostabilizing actions of trehalose will only continue to grow and evolve.

1.2.3. Applications in the Biopreservation Field

Trehalose was first used in the biopreservation field by Beattie *et al.*, who demonstrated the ability of trehalose to improve recovery of human pancreatic islets after cryopreservation.[23] Following that report, trehalose has been shown to enhance the survival of many cryopreserved cells, including fibroblasts, keratinocytes, oocytes, and hematopoietic stem cells.[51; 52; 53; 54] In addition, trehalose has been shown to effectively improve the survival of a number of cells following drying, including platelets,[55] stem cells,[56] and fibroblasts.[57] These studies have demonstrated two important points. First, trehalose needs to be present on both sides of cell membrane to afford maximum protection. Also, an important advantage of using trehalose as an intracellular CPA is that much lower concentrations are needed to achieve similar levels of cryo- and lyoprotection compared to traditional permeating CPAs. Although most literature shows the importance of achieving a threshold level of intracellular trehalose in the millimolar range of concentrations before substantial beneficial results are seen, this threshold level is highly specific for each cellular system. Combined with low trehalose toxicity, low concentrations of trehalose needed to confer protection provide a potential benefit of infusing freeze-thawed or rehydrated cells directly into patients without the cumbersome steps involved in the removal of traditional CPAs.

Although trehalose was first associated with anhydrobiosis and biostabilization over 50 years ago,[23] the fact that mammalian cell membranes are impermeable to trehalose has greatly hindered its application in the biopreservation field. A number of innovative approaches have been used in the past to facilitate intracellular accumulation of trehalose. Beattie *et al.* successfully introduced trehalose into pancreatic islets by taking advantage of the high permeability of membranes at the membrane phase transition, along with the addition of DMSO.[23] Toner's group described the introduction of genetically engineered mutant of a pore protein (α-hemolysin) to overcome the impermeability of mammalian cell membranes.[52; 57] Crowe's group focused on thermal poration, utilizing fluid-phase endocytosis and membrane phospholipid phase transition temperatures to load intracellular trehalose.[55; 58] Guo *et al.* have induced expression of genes that encode for trehalose-6-phosphate synthase in human fibroblasts, which resulted in increased desiccation tolerance of these cells.[44] Other membrane permeablilizing approaches for cytoplasmic trehalose accumulation have been described, including microinjection,[53] $P2X_7$ receptor poration,[59] electroporation,[60] and permeabilization by high-intesity femtosecond laser pulses.[61]

However, all these membrane permeabilization techniques have been shown to have multiple technical and practical limitations. For example, genetically engineered bacterial pore-forming toxins may induce potential cytotoxic and immunogenic effects;[57; 61; 62] electroporation often results in irreversible membrane and cell damage;[62; 63; 64] thermal poration is restricted to mammalian cells undergoing a distinct thermotropic phase transition;[55; 62; 65] microinjection is inherently a very tedious procedure that is limited to a small population of cells;[53; 62] and although effective, the cost and technical expertise needed to perform permeabilization by high-intensity femtosecond laser pulses are a major factor hindering integration of this technology into the biological sciences.[61] Therefore, further research into membrane permeabilization techniques is critical for more effective adoption of trehalose and other nonpermeating intracellular CPAs in biopreservation applications.

1.3. RBC Cryopreservation

1.3.1. Why Preserve RBCs?

The development of effective RBC biopreservation techniques that maintain ex vivo RBC viability and function represents the foundation of modern blood banking. The ability to preserve the integrity of RBCs outside the native environment for extended periods has not only separated blood donors and recipients in space and time, but has also made it possible for blood banks to provide safe, high-quality blood products in an efficient manner. The major force driving the field of RBC biopreservation is the enormous clinical need for RBC products. RBC transfusions save lives by increasing RBC mass in patients that have low oxygen–carrying capacity due to increased RBC loss (traumatic/surgical hemorrhage), decreased bone marrow production (aplastic anemias), defective hemoglobin (hemaglobinopathies and thalassemias), and decreased RBC survival (hemolytic anemias). However, as with any medical procedure, the patients face potential risks associated with hemotherapy, including immune- and nonimmune-mediated transfusion-related adverse reactions. Maintaining the quality and safety of RBCs delivered to the patient, as well as the overall clinical use of blood products, requires effective techniques for preserving RBC viability and function.

As the goal of RBC biopreservation is to provide viable and functional RBCs for patients requiring a blood transfusion, knowledge of RBC physiology is essential to assess the effectiveness of a biopreservation approach, as well as the *in vitro* and *in vivo* quality of transfused RBCs. Derived from pluripotent stem cells in bone marrow through a maturation process called erythropoiesis, mature RBCs are biconcave disks approximately 7.2 μm in diameter, 1.5 to 2.5 μm thick, with a mean volume of 90 fL.[66] The RBC maturation process involves 6 morphologically distinct developmental stages: rubiblast, prorubicyte, rubicyte, metarubicyte, reticulocyte, and mature RBC. With each successive developmental stage, there is a reduction in cell volume, condensation of chromatin, loss of nucleoli, decrease in the nucleus, RNA, mitochondria, and an increase in hemoglobin synthesis, resulting in a mature RBC, which lacks a nucleus and organelles.[66] During its 120-day lifespan, the red blood cell meets the extraordinary demands on deformability and structural stability, which are necessary for it to optimally perform its function of oxygen delivery. The biconcave cell shape is important for both cell flexibility and function, as the specifically high surface area to volume ratio is related to RBC ability to move through microcapillaries, thus supplying the tissues with oxygen. During a normal life span, circulating RBCs undergo metabolic and physical changes associated with the process of senescence, and are recognized and removed by the fixed macrophages in the reticuloendothelial system.[66] Although RBC physiology has been exhaustively investigated, there are still many crucial questions left unanswered, such as the actual biochemical structure of the RBC membrane, and the physiological mechanism of RBC shape regulation, senescence, and destruction.

Current RBC blood banking heavily relies on hypothermic (liquid) storage in additive solutions. Although hypothermic storage slows down the RBC biochemical processes that result in nutrient depletion and accumulation of cell wastes, cellular metabolism is not completely suppressed at these temperatures. Therefore, additive solutions are only able to extend *ex vivo* storage of RBCs to several weeks before RBC quality deteriorates. There still exists a need for a preservation technology that not only delays the deterioration of quality, but fully preserves RBC viability and function for extended storage periods. Long-term RBC preservation would allow maximum utilization of donated blood by reducing the number of products that are discarded due to outdating. It would also improve inventory management by allowing stockpiling of desired units. Furthermore, it would allow maintaining inventory of RBC units with unpredictable needs, such as autologous units and units with rare and unique RBC phenotype. Cryopreservation can offer all of these benefits.

1.3.2. Clinical RBC Cryopreservation

Cryopreservation of RBCs for transfusion is one of the most important applications of cryobiology research. The idea of cryopreserving RBCs and storing them at ultra-low subzero temperatures, so that all metabolic and biochemical reactions cease, has been around for at least 150 years.[3] Basil Luyet, who used ultra-rapid freezing and thawing to recover intact RBCs, is considered to be a pioneer of RBC cryopreservation.[67] However, only small volumes of RBCs could be preserved using Luyet's rapid-freeze technique. Following the serendipitous discovery of the cryoprotective properties of glycerol by Polge, Smith and Parkes in 1950,[15] the entire field of cryobiology rapidly expanded. Audrey Smith was the first to report that freezing RBCs with 15 % glycerol prevented hemolysis[68]. Shortly thereafter, Mollison and Sloviter reported the first successful transfusion of frozen RBCs, with apparent *in vivo* survival comparable to liquid-stored controls.[69] These important findings have led to the development of effective and safe procedures for clinical RBC cryopreservation, which can be separated into three major groups: the cytoglomeration technique, the high glycerol/slow freeze technique, and the low glycerol/rapid freeze technique.

The inability to efficiently add and remove glycerol from RBCs under sterile conditions prohibited wider use of frozen blood in a clinical setting until the late 1950s when Tullis *et al.* described the "Cohn fractionator", one of the first continuous-flow centrifuges to both glycerolize and deglycerolize the cells prior to clinical use. In the early 1960's Huggins applied some principles of the Cohn fractionator to develop the cytoglomeration technique for clinical freezing of RBCs. This technique allowed for removal of the cryoprotectant in a closed system by aggregating RBCs in low-ionic strength, low-pH sugar solutions, then suspending the RBCS in an isotonic medium.[70] Although the cytoglomeration was at first favourably received by many hospital blood banks that set up their own frozen blood processing centers, this technique is rarely used today due to the commercial unavailability of the cytoglomeration equipment and supplies.

In the early 1970s, Meryman and Hornblower greatly simplified the Tullis continuous-flow centrifugation process by freezing the RBC unit in the original collection bag and deglycerolizing by dilution with a series of saline solutions of gradually decreasing osmolality.[71] This technique used 40 % (wt/vol) glycerol in conjunction with slow cooling (~1 °C/min), storage at -80 °C, and rapid thawing in a 37 °C waterbath, and became widely known as the high glycerol/slow freeze method. In the 1970s and 1980s, Valery *et al.* from the U. S. Naval Blood Research Laboratory had extensively evaluated the high glycerol/slow freeze method and introduced the idea of coupling rejuvenating solutions with RBC cryopreservation.[72; 73] Currently, Meryman's high glycerol/slow freeze method is still the clinical method of choice for RBC freezing in North America.

In 1968 Rowe and colleagues in the New York Blood Center applied the kinetic approach first suggested by Luyet, and developed a low glycerol/rapid freeze technique for RBC cryopreservation.[74] This approach involved addition of low concentrations (15 % - 20 %) of glycerol, rapid cooling (<100 °C/min) by immersion into liquid nitrogen, storage in liquid nitrogen (−196 °C) or nitrogen vapor (−165 °C), with rapid thawing in a 42 °C to 45 °C water bath. Blood frozen by this method also met with clinical success, and is still used routinely by European blood banks.

Another approach to RBC cryopreservation involves the use of nonpermeating CPAs, such as hydroxyethyl starch (HES), polyvinylpyrrolidine

(PVP), dextran, and serum albumin. The major advantage of using nonpermeating CPAs is that the thawed RBC units could be transfused immediately. In addition, RBC osmotic stress associated with the addition and removal of glycerol could be circumvented. For example, Sputtek *et al.* have done extensive studies evaluating the use of HES as a nonpermeating CPA for clinical RBC cryopreservation. They reported acceptable post-thaw *in vitro* RBC quality and no adverse reactions in patients receiving autologous transfusion of HES-cryopreserved RBCs, which were washed after thawing.[75] However, nonpermeating CPAs have been shown to stabilize RBC membrane only upon thawing, so the hemolysis is delayed, occurring after the RBCs are transfused.[76] In addition, the safety of infusing high concentrations of these CPAs is questionable.[76] Therefore, nonpermeating CPAs have never been licensed for clinical RBC cryopreservation.

The glycerol-based methods still remain the standard of practice for maintaining an adequate inventory of RBCs with unpredictable patient needs and logistical problems.[77] Current standards stipulate that for deglycerolized RBCs to be suitable for transfusion, deglycerolization and washing must ensure adequate removal of the cryoprotective agents and minimal hemolysis in the supernatant solution.[77] Moreover, post-thaw recovery must be at least 80 % after the deglycerolization process, and the *in vivo* viability of the RBCs must be at least 75 % 24 hours after transfusion.[77] If RBC deglycerolization is performed in a traditional open system, thawed RBCs can be stored hypothermically (1-6 °C) for no more than 24 h due to potential bacterial contamination.[77]

1.3.3. *Shortcomings of Traditionally Cryopreserved RBCs*

Although cryopreservation offers an enormous benefit of long-term storage of RBC units, there are multiple shortcomings related to the current glycerol-based RBC cryopreservation techniques. The labour-intensive, technically-demanding nature of the conventional cryopreservation procedures results in a high cost of the cryopreserved RBC products. Hess estimated that a deglycerolized RBC unit costs about four times as much as the leukoreduced RBC unit from which it was made.[76] In addition, RBC deglycerolization which still requires about 40 min and specially trained technologists makes cryopreserved RBCs economically unfeasible for routine clinical use. The limited 24-h shelf life of deglycerolized units is another disadvantage, but it is being addressed by the recently-licensed closed-system cell washer.[78] In addition to disadvantages related to cryopreservation techniques, current regulations do not specifically address the quality of deglycerolized RBCs, only recovery and sterility.[79; 80] Although many studies have shown that *in vitro* parameters of deglycerolized RBCs are comparable to hypothermically stored RBCs, the 24 h *in vivo* survival of cryopreserved RBCs is significantly lower (while still meeting the minimum 75 % stipulated regulation).[80; 81; 82; 83; 84; 85] Current research suggests that more subtle membrane changes can induce post-transfusion erythrophagocytosis and therefore act as potentially significant predictors of RBC *in vivo* survival and function.[86; 87; 88; 89; 90; 91; 92; 93; 94] Further research is required to investigate the effects of cryopreservation on these novel markers of RBC injury.

These multiple disadvantages translate into low frozen RBC inventories both in North America and Europe.[95; 96; 97; 98] However, as Hess states, cryopreserved RBCs are here to stay.[76] Cryopreservation is the only technology that enables RBCs to be stored safely for decades. Improved clinical RBC cryopreservation will be driven by research in the cryobiology field. Integration of current cryobiology research with blood banking practices offers enormous potential for future improvements. Improved cryopreservation techniques will facilitate the use of frozen RBCs, by improving the quality of thawed RBC products and making them easier to use in clinical situations.[76]

1.4. Thesis Approach

Recent advances in cryobiology research on nonpermeating intracellular CPA trehalose might alleviate problems associated with the traditional glycerol-based cryopreservation methods used in transfusion medicine. In contrast to glycerol, trehalose is not toxic, and the concentrations required for cryoprotective effect have been shown to be significantly less.[51; 52; 53; 54] Therefore, the introduction of intracellular trehalose would circumvent the necessity for the expensive and tedious deglycerolization procedure, resulting in cryopreserved RBCs available for transfusion immediately upon request. However, the RBC membrane impermeability to trehalose provides a significant challenge. The previously proposed membrane permeabilization techniques would not be acceptable for clinical transfusion medicine.

This thesis proposes an alternative approach to loading intracellular trehalose – liposomes. Liposomes are microscopic, spherical, synthetic vesicles, composed of lipid bilayer enclosing an aqueous core. They were first described in 1965 by Bangham *et al.*, who noticed that when phospholipids are placed in an aquous medium, the hydrophilic interaction of the phospholipid head groups results in the formation of heterogeneous multilamellar vesicles.[99] At first, liposomes were used to study biological membranes. As liposomes can be synthesized to conform to many sizes and compositions, this enormous versatility in the physical parameters of liposomes created an attractive potential for a wide range of applications. Practical applications of liposomes as delivery vesicles of both hydrophilic and hydorphobic biomolecules emerged in the late 1970s. Today, liposomes are used as biocompatible carriers of drugs, peptides, proteins, DNA, for pharmaceutical, biochemical, and diagnostic purposes.[100; 101; 102; 103] In addition, they are considered to be a useful model, reagent and delivery tool in many scientific disciplines, including biology, physics, colloid science, cosmetic and food industry.[100; 101; 104]

Liposomal delivery could circumvent many difficulties associated with the previously mentioned permeabilization techniques. Liposomes are nontoxic, non-immunogenic, and FDA-approved as delivery vesicles.[100; 104] Furthermore, liposomal delivery does not require specialized equipment, extensive cellular manipulations, or specific technical expertise. In addition to the well-described biostabilizing properties of trehalose, liposomes themselves have been shown to confer cell protection by improving the quality of the plasma membrane when cells are subjected to stress conditions, such as lyophilization, high-pressure and hypotonic conditions.[105; 106; 107] Stress conditions are also present during ice formation that that accompanies low temperatures, resulting in cell cryoinjury. Similar to trehalose, the effect of liposomes on cell response to freezing requires further investigation. Therefore, by incorporating liposome, cryobiology and transfusion medicine research, this thesis is undertaking a multidisciplinary approach to advance the science of RBC cryopreservation.

1.5. Hypothesis and Thesis Objectives

This thesis will test the hypothesis that liposomal delivery can be used as a permeabilization strategy for the intracellular accumulation of trehalose with minimal injury to RBC membranes, providing a novel approach for RBC cryopreservation. The main objective of this thesis is to increase our understanding of the effects of liposome-RBC interaction on RBC response to freezing, in order to guide a liposome-based approach to improved RBC cryopreservation for transfusion medicine applications. This thesis consists of systematic studies with five specific research aims (SRAs):

SRA 1: *To evaluate the quality of conventionally cryopreserved RBCs using novel indicators of RBC membrane lesion and to correlate them to traditional markers of RBC injury (Chapter 2)*

SRA 2: *To develop and characterize a liposome-based method for the intracellular delivery of trehalose (Chapter 3)*

SRA 3: *To develop an understanding of the mechanism(s) by which trehalose-containing liposomes interact with human RBCs (Chapter 4)*

SRA 4: *To examine the effects of various experimental parameters on liposome-RBC interaction and on RBC membrane quality (Chapter 5)*

SRA 5: *To investigate the effects of liposome treatment on RBC response to freezing and membrane post-thaw quality using conventional and novel indicators of RBC membrane lesion (Chapter 6)*

Following through investigation and discussion of each of these research objectives, this thesis will conclude with a discussion of the presented research, its broad implications and future directions to be explored.

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Chapter 2*

The Effects of Cryopreservation on Erythrocyte Microvesiculation, Phosphatidylserine Externalization and CD47 Expression

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2.1. Introduction

The idea of storing RBCs at ultra-low subzero temperatures to halt all metabolic and biochemical reactions, has been around for at least 150 years.[1] Although additive solutions have extended the liquid storage time of RBCs to several weeks, there is still a need for technology that provides long-term product storage. Since the 1950s, cryopreservation has been used in transfusion medicine for long-term storage of RBCs from donors with rare or unusual phenotypes.[1; 2; 3] In addition, stockpiling a frozen RBC inventory is beneficial for military deployment, or in situations where an unpredictable need for RBC units exists.[1; 2; 3; 4] In Canada, RBCs frozen in 40 % (wt/vol) glycerol are currently approved for storage at -80 °C for up to 10 years.[2] If RBC deglycerolization is performed in a traditional open system, thawed RBCs can be stored hypothermically (1-6 °C) for no more than 24 h due to potential bacterial contamination.[2]

The quality, safety and therapeutic efficacy of RBCs cryopreserved in a high concentration of glycerol have been extensively evaluated over the past four decades, most notably by Valeri *et al.* at the U.S. Naval Blood Research Laboratory (NBRL).[3; 5; 6; 7; 8; 9; 10] In these studies, RBCs were collected in anticoagulants, such as ACD, CPD, or CPDA-1 with or without additive solutions[2] such as AS-1, AS-3 and AS-5, and frozen using several high glycerol methods, including those developed by Cohn,[11] Huggins,[12] Meryman[13] and the NBRL.[14] Numerous variables in RBC cryopreservation were evaluated in these studies, including the methods of collection, the temperature and length of frozen storage, the anticoagulants, cryoprotectants and resuspension media, as well as the incidence of breakage of the freezing containers. These studies provided valuable conclusions for optimizing the high-glycerol method for clinical RBC cryopreservation, including: frozen RBCs should be stored between -65 °C and -90 °C; RBCs can be frozen up to 37 years and maintain their quality; RBCs collected in polyvinylchloride bags should be stored frozen in a rigid cardboard box to prevent bag breakage; and the horizontal -80 °C freezers maintain storage temperature better than the upright -80 °C freezers. These studies also confirmed that RBCs cryopreserved in a high concentration of glycerol meet the AABB standards of minimal hemolysis in the supernatant solution, post-thaw recovery of at least 80 % after deglycerolization, and *in vivo* RBC viability of at least 75 % 24 h after transfusion.[2]

However, current regulations do not specifically address the quality of RBCs.[10; 15] Conventional biochemical and biomechanical assays have been used to further examine the *in vitro* quality of cryopreserved RBCs, including percent hemolysis, ATP, 2,3-DPG, osmotic fragility, and extracellular potassium levels, while the *in vivo* safety and therapeutic efficacy was established by determining the 24 h post-transfusion survival of autologous chromium-labeled RBCs.[3; 5; 6; 7; 8; 9; 10] These studies have shown that *in vitro* quality parameters of deglycerolized RBCs are comparable to hypothermically stored RBCs, while the 24 h *in vivo* survival of cryopreserved RBCs was lower, although it met the minimum 75 % stipulated regulation. There is strong evidence in the current literature that RBC viability, defined by post-transfusion RBC survival, is closely related to the structural and metabolic status of the RBC membrane.[16; 17; 18; 19; 20] Conventional measures of RBC membrane integrity are percent hemolysis and extracellular potassium levels. However, increased membrane permeability to hemoglobin and cations is considered to be one of the last events before cell lysis.[18; 21] Current research suggests that more subtle membrane changes can induce post-transfusion erythrophagocytosis and therefore act as potentially significant predictors of RBC *in vivo* survival and function.[18; 22; 23; 24; 25; 26; 27; 28; 29]

With the recent national adoption of the buffy coat blood component production method[30] by Canadian Blood Services (CBS), validation of the processing of cryopreserved / deglycerolized RBCs from leukoreduced CPD/SAGM RBCs using the high-glycerol Meryman method was required. In addition to assessing the quality of this new CBS product using conventional *in vitro* biochemical and biomechanical assays, RBCs were evaluated for novel markers of membrane injury, including: exposure of PS on the outer leaflet of the cell membrane,[18; 27; 31; 32] decreased expression of integrin-associated CD47 antigen on the cell surface,[22; 23; 33; 34] and loss of membrane phospholipids through microvesiculation.[18; 24; 29; 35]

The phospholipids of RBCs are distributed asymmetrically in the bilayer of membrane, with choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (SM) localized mainly in the outer monolayer of the membrane, whereas the aminophospholipids phosphatidylethanolamine (PE) and PS are found in the inner monolayer. Phospholipid asymmetry is essentially conserved throughout the lifespan of RBC by means of energy-dependent transfer.[31; 36] Similar to apoptosis in nucleated cells, PS exposure from randomization of the asymmetric distribution of membrane is a characteristic feature of RBC senescence, leading to erythrophagocytosis. [18; 25; 27; 31; 32] Increased erythrophagocytosis is also related to decrease in the expression of CD47 antigen on RBC membranes.[22; 23; 33; 34] CD47 is an integrin-associated glycoprotein that is ubiquitously expressed, acting as a marker of self.[33; 34; 37] The interaction between the CD47 molecules on normal RBCs and signal regulatory protein α on macrophages sends an inhibitory signal to macrophages that protects RBCs from phagocytosis. [22; 23; 26; 33; 34] Additional characteristic of senescent RBCs leading to erythrophagocytosis is membrane loss through microvesiculation. Lipid loss from the RBC membrane happens both *in vivo* and during *in vitro* storage through the blebbing of microvesicles from the tips of echinocytic spicules and is balanced with respect to both cholesterol and phospholipids.[16; 17; 29; 38; 39] The transverse migration of PS is generally coincident with membrane microvesiculation, which is thought to result from a transient overload of the outer leaflet at the expense of the inner one.[28; 29; 40; 41; 42] When the cytoskeleton is no longer able to counteract the surface tension, shedding of membrane microparticles takes place.[28; 29] RBC membrane microvesicles not only lead to premature erythrocyte clearance, but also carry accessible PS, membrane antigens including adhesion proteins, enabling them to participate in blood coagulation, cell adhesion, and inflammation.[28; 29; 40]

Although the significance of PS externalization, decreased CD47 expression and membrane microvesiculation in relation to the quality and safety of RBC concentrates is not well understood, recent studies have identified these markers to be potentially significant determinants of *in vivo* RBC survival and function, and may also act as harmful immunomodulators.[18; 22; 23; 24; 25; 26; 27; 28; 29] Changes in RBC surface expression of PS and CD47 and increased membrane microvesiculation have been recently documented as features of the hypothermic storage lesion.[18; 22; 23; 24; 25; 26; 27; 28; 29] However, these markers have never been examined in cryopreserved / deglycerolized RBCs. In view of the potential immunomodulatory and pathophysiologic significance of these factors, the purpose of this study was to assess the effects of cryopreservation, 24 h post-deglycerolization hypothermic storage and pre-freeze storage length on membrane microvesiculation, phosphatidylserine externalization and CD47 expression of leukoreduced CPD/SAGM RBCs, as well as to correlate these measures to traditional RBC biochemical and biomechanical quality indicators. The purpose of this thesis is to investigate a new approach to RBC cryopreservation, involving trehalose and liposomes. This chapter will establish a baseline of RBC post-thaw quality when RBC are cryopreserved using a traditional high-glycerol method, before a new approach to RBC cryopreservation is further investigated in following chapters.

2.2. Materials and Methods

The experimental design of this study is illustrated in Figure 2.1.

2.2.1. RBC Processing[*]

Whole blood (450 ± 50 mL) was collected from healthy volunteers according to Canadian Blood Services (CBS) protocols. Leukoreduced CPD/SAGM packed RBC units were produced at the CBS Edmonton Centre using the standard buffy coat procedures.[2] In the buffy coat production system, packed RBCs are derived from whole blood collection in citrate-phosphate-dextrose (CPD) anticoagulant, which is centrifuged and the plasma and the buffy coat removed. The RBCs are subsequently re-suspended in saline-adenine-glucose-mannitol (SAGM) additive nutrient solution and are then leukoreduced by filtration.[2] These units were hypothermically stored (1-6 °C) prior to freezing for 2-3 d (n=8) or 13-14 d (n=18) post-collection.

Meryman's high glycerol method was used to freeze the RBC units.[13] Briefly, this method involves adding Glycerolyte 57™ solution (Baxter, Deerfield, IL) to a unit of RBCs to achieve a final glycerol concentration of approximately 40 % (wt/vol), placing the glycerolized unit in a metal container for freezing, freezing at rate of ~1 °C/min, and subsequent storage at −80 °C. All units were stored at −80 °C for at least one week (7 – 13 d) before thawing. The RBC units were thawed at 36 - 38 °C in a mechanically agitated water bath and washed with 150 mL of 12 % NaCl, 2 L of 1.6 % NaCl, and 1 L of 0.9 % NaCl / 0.2 % dextrose solution (Baxter Healthcare Corporation, Deerfield, IL) by serial centrifugation in a cell washer (COBE 2991, Model 1, IBM, Princeton, NJ). Post-thaw testing was performed immediately after deglycerolization and required about 3 h. The deglycerolized RBC units were hypothermically stored (1-6 °C) for 24 hours, after which RBC *in vitro* quality assessment was repeated. Three leukoreduced CPD/SAGM RBC units hypothermically stored (1-6 °C) for either 3-4 d or 14-15 d were used as unfrozen RBC controls.

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[*] RBC processing was performed by CBS staff at the Edmonton Centre using standard operating procedures.

2.2.2. RBC Quality Assessment

In vitro RBC quality was assessed pre-freeze (2-3 d or 13-14 d post-collection), post-thaw and 24 h post-thaw by both conventional biochemical / biomechanical assays, as well as more novel flow cytometry analysis.

2.2.2.1 Conventional RBC Quality Assessment[*]

RBC *in vitro* quality was examined using conventional biochemical and biomechanical assays including percent hemolysis, ATP concentration, extracellular potassium levels, RBC indices and morphology.

Hemolysis represents the breakdown of the red blood cell membrane and subsequent release of hemoglobin. Percent hemolysis was determined by comparing the supernatant hemoglobin to total hemoglobin concentrations, using the cyanmethemoglobin method, which is considered the reference method for hemoglobin determination.[43] In this method, blood is diluted with Drabkin's reagent. Most forms of hemoglobin are then converted to the stable pigment cyanmethemoglobin (HiCN). The reaction takes place in two steps: hemoglobin is oxidized to methemoglobin, which then reacts with cyanide to form HiCN. HiCN absorbs light maximally at 540 nm and the measured absorbance is directly proportional to the hemoglobin concentration of the sample. Since HiCN solutions strictly follow the Lambert-Beer's law[44], the concentration of hemoglobin in a solution may be calculated directly from the absorbance of the solution using the following equation[45]:

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[*] Conventional RBC Quality Assessment was performed jointly by Jelena Holovati, Kenneth Wong and Jarret Webster. Kenneth Wong and Jarret Webster work as research technologists in the Acker Research Lab.

$$c = \frac{A_{540} \times M \times F}{\varepsilon_{540} \times l \times 1000} \qquad \text{(Eq. 2.1)}$$

where c = concentration of hemoglobin (g/L)

A_{540} = absorbance of the solution at 540 nm

M = molecular mass of hemoglobin monomer (16114.5 g/M)

F = dilution factor of sample preparation

ε_{540} = millimolar absorptivity of HiCN at 540 nm (11.0 $cm^{-1} \cdot mM^{-1}$)

l = light path (cm)

Once supernatant and total hemoglobin values have been determined, the % hemolysis is calculated from the following equation[46]:

$$\%\ hemolysis = \frac{(100 - Hct) \times Hb_S}{Hb_T} \qquad \text{(Eq. 2.2)}$$

where Hct = hematocrit (%)

Hb_S = supernatant hemoglobin concentration (g/L)

Hb_T = total hemoglobin concentration (g/L)

The percent hemolysis is therefore ratio of supernatant hemoglobin to total hemoglobin. The hematocrit in the calculation accounts for the volume of the supernatant in the specimen. Commercial tri-level hemoglobin solutions (low, mid-range and elevated) were used as controls (Stanbio Laboratory, Boerne, USA).

RBC ATP concentrations were measured in deproteinized extracts by an enzymatic method, using a commercially available kit (Rolf Greiner BioChemica, Flacht, Germany). RBC aliquots were mixed with a trichloroacetic acid solution to precipitate blood proteins and centrifuged to obtain a clear supernatant. ATP from the sample is used in 2 chemical reactions which ultimately convert glucose into 6-phoshphogluconate and NADH. The amount of NADH produced is directly proportional to the amount of ATP originally present in the test sample and can be measured spectrophotometrically at 340 nm. The concentration of ATP may be calculated directly from the absorbance of the solution using the following equation:

$$ATP(\mu mol/dL) = \frac{\Delta A \times V \times F \times 100}{\varepsilon_{340} \times v \times d} \qquad \text{(Eq. 2.3)}$$

where ΔA = (absorbance of the sample solution at 540 nm) – (absorbance of the blank solution at 540 nm)

V = total volume of the sample

F = dilution factor of sample preparation

ε = extinction coefficient of NADH (6.3 @ 340nm)

v = sample volume used in ATP assay

d = light path (cm)

Following calculation can be used to convert ATP concentration from μmol/dL to μmol/gHb:

$$ATP(\mu mol/gHb) = \frac{ATP(\mu mol/dL)}{10 \times Hct(L/L) \times Hb(g/L)} \quad \text{(Eq. 2.4)}$$

Ion selective potentiometry was used to measure extracellular potassium levels (Synchron LX Systems, Beckman, Coulter, Fullerton, CA). RBC samples were centrifuged, supernatants extracted and sent to the University of Alberta Hospital Clinical Chemistry Laboratory for quantitative determination of potassium. University of Alberta Hospital Clinical Chemistry Laboratory routinely analyzes high volume of biological fluid samples for potassium concentration. The Synchron LX System contains potassium ion selective electrode in conjunction with a sodium reference electrode to detect changes in the electron potential, which allows the calculation of potassium ion concentration in the sample. Synchron LX Aqua 1, 2 and 3 solutions were used as controls, while Synchron LX ISE RefEl solution was used as a control reagent (Beckman, Coulter, Fullerton, CA).

Determination of RBC indices gives valuable information that helps to characterize RBCs. The RBC indices consist of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), which were determined using a Coulter automated cell counter (Coulter AcT, Beckman Coulter, New York, NY). As RBCs in suspension pass through the Coulter aperture, they displace their own volume of electrolyte, momentarily increasing impedance of the aperture, which results in a pulse that is directly proportional to the cell volume. In addition, a known volume of the RBC suspension is passed through the aperture, so a count of the number of pulses can yield the concentration of RBCs in sample. The instrument computes hematocrit value by summing the electronic volume of RBCs, while HiCN method is used for determining hemoglobin concentration. MCV is determined by measuring the average volume of individual RBCs and is expressed in fL. MCH is the average weight of hemoglobin in the RBC, expressed in absolute units (pg) and computed by dividing hemoglobin concentration (g/L) by RBC count (RBC/L). Finally, MCHC is the average concentration of hemoglobin in each individual RBC, expressed as a percentage and computed by dividing hemoglobin concentration by the hematocrit. Coulter 4C-ES Low, Normal and High controls (Beckman Coulter, New York, NY) were used to confirm and monitor instrument accuracy and precision by providing measurements for counting, sizing and hemoglobin determination.

RBC morphology was assessed by staining the cells with a Hema 3 staining kit (Fisher Diagnostics, Middletown, VA) and examining cells microscopically at 100x magnification. Three trained lab technologists graded the appearance of RBCs by examining 100 cells and assigning a morphological index according to the six stages of disk to sphere RBC shape transformation (progressive spheroechinocytosis).[47] The percentage of each cell type was multiplied by a factor assigned to each cell type: smooth disc (1.0), crenated disc (0.8), crenated discoid (0.6), crenated spheroid (0.4), crenated sphere (0.2) and smooth sphere (0.0). The morphological index for each RBC sample was then calculated by adding these products (Figure 2.2).

2.2.2.2. *RBC Quality Assessment Using Flow Cytometry*

Flow cytometry was used to examine RBC microvesicle formation, membrane phosphatidylserine externalization and CD47 expression. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific, multi-parameter data from particles suspended in a sheath of phosphate buffered saline fluid. RBCs (10^6/mL) suspended in a binding buffer (150 mM NaCl, 10 mM HEPES, 2 mM $CaCl_2$, Sigma-Aldrich, St. Louis, MO) were labelled with PE-conjugated IgG anti-CD47 and Cy5-conjugated annexin V (BD Biosciences Pharminogen, San Jose, CA) according to manufacturer's instructions. Samples were analyzed on a FACSCalibur flow cytometer (BD Sciences, San Jose, CA) equipped with a 488 nm argon laser and computer software (CellQuest BD Sciences, San Jose, CA) within 1 h of staining. 20,000-25,000 gated events were collected for flow cytometry analysis. A region around the RBC population was set using the unfrozen RBC controls, while 400 nm standard latex beads (Bangs Laboratories Inc., Fishers, IN) were used to set gating for RBC microvesicle population on a forward scatter (FSC) versus side scatter (SSC) plot with logarithmic scales.[23; 35] RBC- and microparticle-associated fluorescence intensity was measured in the FL-2 channel for PE-CD47 (excitation 485 nm, emission 575 nm), and in the FL-4 channel for Cy5-annexin V (excitation 645 nm, emission 680 nm). Positive, negative and isotype flow cytometry RBC controls for PS and CD47 were prepared as described by Stewart et al.[22] Briefly, positive controls were prepared by treating RBCs with N-ethylmaleimide (NEM) and calcimycin, which induces PS exposure and RBC antigen shedding. Negative controls were untreated RBCs. PE conjugated antibody of the same IgG subclass (IgG1) as that of the PE-anti-CD47 was used as a background isotype control to account for any non-specific antibody binding.

2.2.2.3. Additional Post-thaw RBC Quality Assessment[*]

In addition to the above described *in vitro* RBC assays, additional quality assessments were performed post-thaw according to CBS standard operating procedures. The assessment included calculation of RBC recovery, measurement of residual glycerol concentration by refractometry[48] (Reichert Refractometer, Depew, NY), measurement of total supernatant hemoglobin (cyanmethemoglobin method[49]), and sterility testing[50] (BacT/ALERT, Becton-Dickenson Diagnostic Systems, Sparks, MD).

2.2.3 Statistical Analyses

The data were analyzed using commercial software (SigmaStat version 10.0, Systat Software, Germany and SPSS version 12.0, Lead Technologies, Charlotte, NC). Results are expressed as mean ± SD unless specified. Due to the small sample size (n=26), nonparametric statistical analysis was performed. The differences between two independent groups at each testing time point were examined using the Mann–Whitney U test, whereas the Kruskal-Wallis ANOVA was used to assess the differences in RBC *in vitro* parameters between the pre-freeze, post-thaw, 24 h post-thaw time points and hypothermic controls. Probabilities less than 0.05 were considered significant.

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[*] Additional post-thaw RBC quality assessment was performed by CBS medical laboratory technologists at the Edmonton Centre using standard operating procedures.

2.3. Results

All deglycerolized units showed acceptable post-deglycerolization quality according to CBS standards. Freeze-thaw-wash recovery was greater than 80 % (92 ± 4 %); residual glycerol concentration was less than 1 % wt/vol (0.6 ± 0.2 %); deglycerolized supernatant hemoglobin concentration was less than 0.2 g / RBC unit (0.1 ± 0.04 g / RBC unit); and all deglycerolized units were sterile. No bags broke during processing.

Pre-freeze, post-thaw and 24 h post-thaw conventional *in vitro* quality measures of RBC units from both test groups and hypothermic controls are shown in Tables 2.1 and 2.2. Hypothermic controls were used to help distinguish between RBC injury due to cryopreservation (including the deglycerolization process) and injury due to hypothermic storage alone. There was no statistically significant difference between the pre-freeze RBC quality results and the hypothermic controls for any of the assays performed (Tables 1 and 2). When comparing pre-freeze RBC results with post-thaw and 24-h post-thaw values, it is important to keep in mind that the deglycerolization process involves a series of washings with saline solutions of gradually decreasing osmolality, which results in the removal of a majority of extracellular substances and probably some damaged RBCs.

Percent hemolysis increased over time in both the 2-3 d pre-freeze (Table 2.1) and 13-14 d pre-freeze storage test groups (Table 2.1), as demonstrated by the statistically significant increases in the percent hemolysis values between the pre-freeze, post-thaw, and 24 h post-thaw time points ($p<0.001$ for both groups). Percent hemolysis values were significantly higher in the 13-14 d pre-freeze storage test group compared to the 2-3 d pre-freeze storage group both at the pre-freeze ($p=0.046$) and 24 h post-thaw ($p=0.040$) time points. There were no statistically or biologically significant (biologically significant defined as outside the clinical reference range) changes in the MCV ($p=0.961$), MCH ($p=0.892$) and MCHC ($p=0.794$) values at the three testing time points. ATP levels were not statistically different between the two test groups at the pre-freeze ($p=0.083$), post-thaw ($p=0.196$) and 24 h post-thaw time points ($p=0.102$). Also, there was no significant decrease in ATP levels after deglycerolization and after 24 h of post-thaw hypothermic storage in either of the groups ($p=0.773$ and $p=0.189$). Prolonged pre-freeze storage resulted in a statistically significant increase in extracellular potassium concentration ($p<0.001$). However, as expected, this excess potassium was removed during the deglycerolization process. There were no biologically significant differences in the potassium levels between the two test groups after deglycerolization. The RBC morphology index showed a statistically significant decrease after deglycerolization and after 24 h of post-thaw storage in both test groups ($p<0.001$), reflecting progressive RBC spheroechinocytosis. In addition, the morphology index of RBCs with 13-14 d pre-freeze storage was significantly lower compared to RBCs with 2-3 d pre-freeze storage at all three testing time points (p<0.001).

Figure 2.3 illustrates examples of the FSC versus SSC plots used to set gates around the RBC and microvesicle populations, to examine possible changes in particle size and to obtain the annexin V and CD47 fluorescence intensities of each population. In both test groups, the mean FSC of RBC microvesicles did not significantly change after cryopreservation (p=0.072 in 2-3 d group; p=0.067 in 13-14 d group) and following the 24-h post-thaw period (p=0.062 in 2-3 d group; p=0.064 in 13-14 d group), nor was there a statistically significant difference between the 2-3 d and 13-14 d pre-freeze storage test groups (pre-freeze p=0.064, post-thaw p=0.064, 24 h post-thaw p=0.059).

Changes in PS exposure of the RBC population can be seen in Figure 2.4. For the 2-3 d stored units, there was no statistically significant difference in PS exposure between the pre-freeze, post-thaw and 24 h post-thaw time points (p=0.612). For the 13-14 d stored units, there was no statistically significant difference in PS exposure between the pre-freeze and post-thaw time points (p=0.139). However, following 24 h of post-thaw storage, PS exposure in the 13-14 d stored RBCs was significantly higher compared to pre-freeze levels (p=0.021). The amount of PS exposure was not statistically different between the 2-3 d and 13-14 d stored RBCs at both the pre-freeze (p=0.378) and post-thaw (p=0.224) time points. After 24 h of post-thaw storage a significant increase in PS exposure was seen in the 13-14 d stored units compared to those stored for 2-3 d (p=0.016). In both test groups, the amount of CD47 expression on

RBCs (Figure 2.5) did not significantly decrease after cryopreservation (p=0.059) and following the 24-h post-thaw period (p=0.062), nor was there a statistically significant difference between the 2-3 d and 13-14 d pre-freeze storage test groups (pre-freeze p=0.064, post-thaw p=0.061, 24 h post-thaw p=0.066).

Figure 2.6 illustrates the accumulation of RBC membrane microvesicles in both test groups, which was evaluated by comparing event percentages calculated by dividing the number of events in the microparticle gate by the sum of RBC and microparticle events. Microparticle event percentage was significantly higher in the 13-14 d stored RBC samples compared to the 2-3 d pre-freeze units at the pre-freeze (p<0.001) and 24 h post-thaw (p=0.035) time points, but not immediately after deglycerolization (p=0.216). Furthermore, a statistically significant increase in the microparticle number was seen after 24 h of post-thaw storage in the 13-14 d stored units (p=0.017), but not in the 2-3 d stored units (p=0.059) when compared to the post-thaw values.

In addition to changes in the microvesicle numbers, we examined the changes in the expression of PS and CD47 on the microvesicle surfaces by dividing the population-associated fluorescence intensity by the number of microparticle events (Figure 2.7). For the 2-3 d stored RBC units, while significantly more PS was expressed per microparticle at post-thaw and after 24 h post-thaw storage as compared to pre-freeze values (p<0.001 for both comparisons), the change in PS fluorescence intensity per microparticle between the post-thaw and 24 h post-thaw time points was not statistically significant (p=0.952). In contrast, the microparticles from the 13-14 d stored units showed a statistically significant increase in the expression of PS per microparticle following 24 h of post-thaw storage compared to the pre-freeze and post-thaw time points (p<0.001), while microparticle PS expression did not significantly change after cryopreservation (p=0.611). The PS fluorescence intensity per microparticle was statistically different between the 2-3 d and 13-14 d stored units at pre-freeze (p=0.001) and following 24 h post-thaw storage (p<0.001), but not immediately after deglycerolization (p=0.10). Similar to RBC CD47 expression, microparticle CD47 fluorescence intensity did not significantly change after cryopreservation (p=0.994) and following the 24 h post-thaw period (p=0.533), nor was there a statistically significant difference between the 2-3 d and 13-14 d pre-freeze storage test groups (pre-freeze p=0.054, post-thaw p=0.062, 24 h post-thaw p=0.055, Figure 2.8).

2.4 Discussion

As expected, leukoreduced CPD/SAGM RBCs showed acceptable *in vitro* quality after deglycerolization, according to the conventional biochemical and biomechanical assays. All the post-thaw parameters, including freeze-thaw-wash recovery, residual glycerol concentration, supernatant hemoglobin concentration and sterility passed CBS quality requirements. Although there were statistically significant changes in RBC hemolysis, morphology, ATP and extracellular potassium concentrations after cryopreservation, deglycerolization and following 24 h post-deglycerolization hypothermic storage, reflecting either cryoinjury, removal of extracellular substances during the deglycerolization process, or hypothermic storage lesion, these changes are not likely biologically significant, as the values remained within normal reference ranges.[2; 7; 51; 52] In addition, the conventional *in vitro* quality parameters of deglycerolized RBCs in our study are consistent with previously published literature evaluating the quality of cryopreserved RBCs, even though many protocol steps, including preparation of packed RBCs, anticoagulant / additive solutions used, length of the pre-freeze and frozen storage, and freezing method varied among the studies.[4; 7; 52; 53; 54]

In vivo 24 h survival assays are superior to *in vitro* quality assessment as a direct measurement of RBC viability and therapeutic efficacy. As *in vivo* RBC survival is not a measure of RBC function is complex to carry-out, it is beyond the scope of this study. Novel markers of cellular lesion, including phosphatidylserine externalization onto the outer leaflet of the cell membrane, decreased expression of integrin-associated CD47 antigen on the cell surface, and loss of membrane phospholipids through microvesiculation have been recently shown to be potentially significant predictors of RBC *in vivo* survival and function.[18; 22; 23; 24; 25; 26; 27; 28; 29] Physiological RBC senescence or *in vitro* metabolic and mechanical stresses can lead to disruption of the asymmetric phospholipid distribution of the cell membrane, resulting in PS externalization, which then acts as a signal for erythrophagocytosis by macrophages.[10; 18; 22; 55] Furthermore, PS exposure has been shown to enable RBCs to participate in blood coagulation and cell adhesion.[56; 57] Another signal for erythrophagocytosis is decreased expression of CD47 antigen. Since CD47 in normal RBCs activates signal regulatory protein-□leading to the inhibition of macrophage activation for phagocytosis, injured and/or senescent RBCs lacking CD47 are quickly recognized and removed from circulation.[18; 22; 23; 26; 34] In addition to promoting RBC clearance, this type of RBC membrane lesion is also believed to initiate shedding of membrane microparticles, which are thought to result from a transient overload of the outer membrane leaflet at the expense of the inner one.[27; 28; 29; 58; 59] After having long been considered innocuous cell debris, membrane microparticles have more recently been shown to have physiologic and pathophysiologic significance by playing a role in inflammation, hemostasis, and vascular (dys-)function.[28; 29; 40] Therefore, changes in these markers may explain why the *in vivo* survival of cryopreserved RBCs is generally lower than that of hypothermically-stored RBCs, even though the conventional *in vitro* assays show acceptable quality.[6; 9; 17]

Cryopreservation, deglycerolization and post-deglycerolization hypothermic storage can result in mechanical and biochemical stresses inducing subtle membrane lesions, which are not detected by conventional assays. These lesions, marked by PS externalization, decreased expression of CD47 and membrane microvesiculation, may not only hinder the performance of transfused RBCs, but may also compromise the immune and hemostatic systems, especially in critically ill patients.[18; 22; 23; 24; 25; 26; 27; 28; 29] Our previous research on the utilization of frozen blood showed that the majority (89 ± 3 %) of deglycerolized units issued to hospitals by CBS Edmonton Centre between 1992 and 2002 were required for multiple transfusions to a single recipient.[60; 61]

Therefore, membrane lesions not evaluated by conventional *in vitro* quality assays could play a significant role in RBC post-transfusion survival and function in this patient population, which warrants further study.

Another important determinant of the quality of deglycerolized RBCs is the length of hypothermic (1-6 °C) pre-freeze storage.[2; 3; 51; 62] According to AABB Standards, the hypothermic (1-6 °C) pre-freeze storage of RBC units collected in CPD or CPDA-1 must not exceed 6 days, whereas RBC units collected in additive solutions can be hypothermically stored (1-6 °C) for up to 42 d before cryopreservation.[2] As hypothermic storage of RBCs can induce membrane lesions leading to exposure of phosphatidylserine (PS) onto the outer leaflet of the cell membrane,[18; 27; 31; 32] decreased expression of integrin-associated CD47 antigen on the cell surface,[22; 23; 26; 33; 34] and loss of membrane phospholipids through microvesiculation,[18; 24; 29; 35] prolonged pre-freeze storage may predispose RBCs to further injury during cryopreservation, deglycerolization and post-thaw hypothermic storage. Our previous research on the utilization of frozen blood showed that the average length of time packed RBC units are stored hypothermically (1-6 °C) prior to cryopreservation in CBS Edmonton Centre between 1993 and 2002 is substantially less than that permitted by existing standards (7.3 ± 2.0 d, range 1-40 d), but has continuously increased over the past 10 years (1993-1997 average 5.9 ± 1.8 d; 1997-2002 average 8.6 ± 0.9 d, Figure 2.9).[60; 62] Therefore, in addition to evaluating the effects of cryopreservation on these novel markers of membrane injury, we examined the role of hypothermic pre-freeze storage as a predictor of RBC membrane lesion, by evaluating the difference in quality of RBCs stored hypothermically (1-6 °C) prior to freezing for 2-3 d or 13-14 d post-collection.

Our results showed that there was no significant difference in RBC membrane phosphatidylserine externalization, CD47 expression and microvesiculation between the pre-freeze, immediately post-thaw and hypothermic storage control values, suggesting that the RBC cryopreservation process alone does not induce considerable injury to RBC membranes. This was seen in both 2-3 d and 13-14 d pre-freeze stored RBC units. Therefore, the cryoprotective action of glycerol is effective at mitigating slow-cool freezing injury resulting from RBC exposure to concentrated extra- and intracellular solutions at high sub-zero temperatures during the cryopreservation process, regardless of the pre-freeze storage length. However, the pre-freeze storage length was a significant predictor of RBC injury after 24 h of post-thaw storage, as seen by the statistically significant change in PS exposure and microvesicle accumulation in the 13-14 d hypothermically stored units when compared to pre-freeze values, and statistically significant differences between the 2-3 d and 13-14 d pre-freeze stored units. These data suggest that a two-week pre-freeze storage may lead to subtle membrane lesion that is not immediately seen prior to or post-cryopreservation, but when compounded by the 24 h post-thaw hypothermic storage stress, results in marked injury. Consequently, the permitted hypothermic pre-freeze storage of up to 42 d in additive solutions may result in an even more profound membrane injury of deglycerolized RBCs following the post-thaw hypothermic storage. These data suggest that the current regulations need to be reviewed. More defined criteria limiting the pre-freeze storage length should be established for the cryopreservation process. Additional research is needed to examine whether similar effects are seen with the FDA-permitted two-week post-thaw hypothermic storage of RBCs in AS-3, when functionally closed systems (such as the Haemonetics ACP-215) are used for the glycerolization and deglycerolization processes.

ATP depletion during hypothermic storage has been implicated as a significant perpetrator of RBC membrane PS exposure and microvesiculation.[22; 24; 28; 29] Since the ATP concentrations in our samples remained within the normal reference range, the 24 h post-thaw membrane injury is more likely to be biomechanical than biochemical in nature. The expression of CD47 antigen on RBC membranes and RBC microparticles was not influenced by the pre-freeze storage length, cryopreservation process, or the 24 h post-thaw hypothermic storage length. Significant differences in CD47 expression were not expected in pre-freeze results between the 2-3 d and 13-14 d stored RBC units, as previous studies have shown that hypothermic storage will result in decreased expression of CD47 only after 24 d of storage.[22] However, it is interesting that cryoinjury and osmotic stresses did not cause significant changes in the expression of this antigen on RBC membranes. Therefore, our results suggest that this marker of membrane injury is not as sensitive as PS exposure and membrane microvesiculation, and do not support the use of this marker for cryoinjury.

Flow cytometry is the method of choice for measuring membrane microparticles *ex vivo*, as it enables the analysis of thousands of microparticles in one sample, with the simultaneous determination of multiple markers.[63] However, it is important to keep in mind that although RBC microparticles can vary from 100 to 800 nm in size,[29; 41; 42; 64; 65] current commercially available flow cytometers are not capable of analyzing particles smaller than approximately 300 nm. RBC membrane microvesicles expressed similar injury patterns to RBC membranes (Figures 2.7 and 2.8), as shown by a significant increase in PS fluorescence intensity per microparticle after 24 h of post-thaw storage, compared to pre-freeze values, in both the 2-3 d and 13-14 d stored units. Therefore, RBC membranes with compromised phospholipid asymmetry are not only more prone to microvesiculation, but also result in microvesicles with a greater expression of PS on their surface. The deglycerolization process resulted in a reduction of microvesicles in RBC samples, but the remaining microvesicles were highly expressive of PS on their surface. In addition to PS, RBC microvesicles were also highly expressive of CD47 antigen, although there was no statistically significant difference in CD47 fluorescence expression of microparticles between the test groups and time points. A potential explanation for the increase in microvesicle mean fluorescence intensity could also be an increase in mean particle size, as the centrifugation and washing steps during the deglycerolization process could result in the preferential removal of smaller particles from the supernatant. However, there was no change in microvesicle forward scatter, which suggests that the increase in mean fluorescence values in

Figures 2.7 and 2.8 is most likely due to increased expression of PS and CD47 on the microvesicle surface, rather than increased microvesicle size. As expected, the loss of RBC membrane microvesicles correlated with the progressive RBC spheroechinocytosis seen with morphological examination. However, RBC morphological indices remained within normal reference ranges for all groups.

The clinical implications of our findings warrant further study. Although our data show statistically significant changes in RBC membrane PS exposure and microvesiculation between the time points and test groups, statistical significance is not synonymous with clinical significance and the clinical significance of transfusing these RBCs is unclear. There are no guidelines for transfusion of RBCs with increased levels of PS on their surface, or reference ranges for microvesicle concentration in packed RBC units. As for CD47, it has been suggested that RBCs become susceptible to erythrophagocytosis only if CD47 reduction exceeds 50 %,[56] which is not seen in our data. However, increased levels of PS on the RBC surface may render deglycerolized RBCs more susceptible to clearance from circulation when transfused, explaining why the *in vivo* survival of cryopreserved RBCs is generally lower than that of hypothermically-stored RBCs, even though the conventional *in vitro* assays show acceptable quality.[6; 9; 17] Combined with potentially decreased RBC efficacy, transfusion of RBC membrane microvesicles which are highly expressive of PS and CD47 may result in detrimental immunomodulatory, procoagulatory and microcirculatory effects[18; 22; 23; 24; 25; 26; 27; 28; 29] in recipients of deglycerolized RBC units, particularly if these patients receive multiple transfusions, which seems to be true for recipients of frozen RBCs.[60; 61] Therefore, the results of our study also suggest that more research on the *in vitro* and *in vivo* effects of these novel markers of RBC membrane injury is warranted.

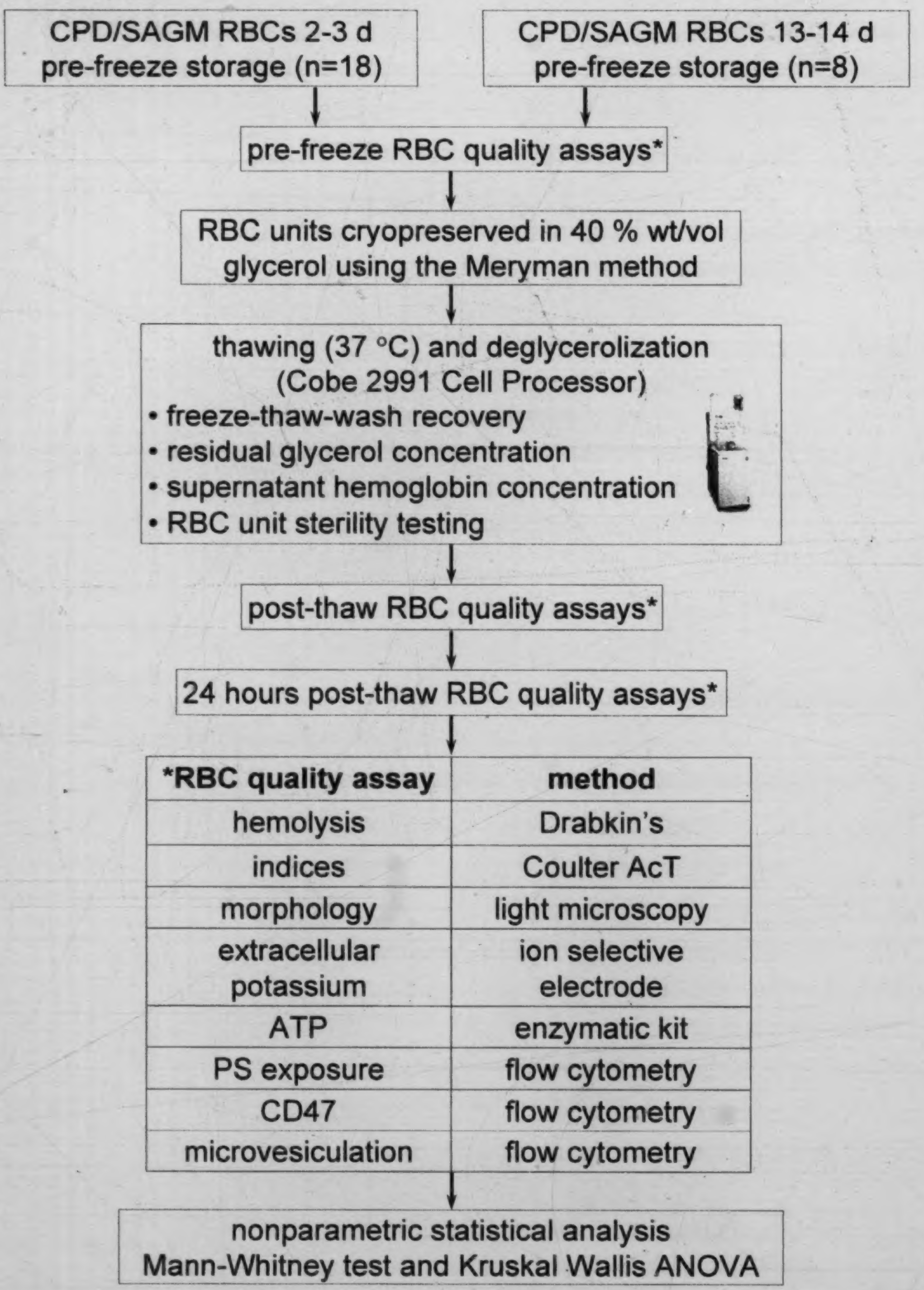
2.5 Conclusion

This study evaluated the effects of cryopreservation, 24 h post-deglycerolization hypothermic (1-6 °C) storage and pre-freeze storage length on novel markers of RBC membrane injury, including microvesiculation, phosphatidylserine externalization and CD47 expression of leukoreduced CPD/SAGM RBCs and correlated these measures to traditional RBC biochemical and biomechanical quality indicators. These novel markers of RBC membrane injury are gaining importance as potentially harmful immunomodulators with pathophysiologic significance.

The results of our study show that deglycerolized leukoreduced CPD/SAGM RBCs show acceptable *in vitro* quality according to conventional biochemical and biomechanical assays, with no biologically significant changes following 24 h of post-thaw storage. High-glycerol cryopreservation alone does not induce PS exposure, loss of CD47 expression and microvesiculation. However, prolonged pre-freeze storage is a strong predictor of PS exposure or microvesiculation occurring after 24 h of post-thaw hypothermic storage. Since prolonged pre-freeze storage can affect RBC membrane asymmetry during the post-deglycerolization storage period, more defined criteria for this parameter should be adopted. More research is required on the *in vitro* and *in vivo* effects of these novel markers of RBC membrane injury.

This chapter also established a baseline of RBC post-thaw quality when RBC are cryopreserved using a traditional high-glycerol method and developed flow cytometry techniques that evaluate subtle RBC membrane injury. The results and techniques presented in this chapter are very valuable for the experimental work in future chapters, which will investigate a new approach to RBC cryopreservation.

Figure 2.1: The experimental design of this study


CPD/SAGM RBCs 2-3 d pre-freeze storage (n=18)
CPD/SAGM RBCs 13-14 d pre-freeze storage (n=8)
pre-freeze RBC quality assays*
RBC units cryopreserved in 40 % wt/vol glycerol using the Meryman method
thawing (37 °C) and deglycerolization (Cobe 2991 Cell Processor)
• freeze-thaw-wash recovery
• residual glycerol concentration
• supernatant hemoglobin concentration
• RBC unit sterility testing
post-thaw RBC quality assays*
24 hours post-thaw RBC quality assays*
nonparametric statistical analysis Mann-Whitney test and Kruskal Wallis ANOVA

***RBC quality assay**	**method**
hemolysis	Drabkin's
indices	Coulter AcT
morphology	light microscopy
extracellular potassium	ion selective electrode
ATP	enzymatic kit
PS exposure	flow cytometry
CD47	flow cytometry
microvesiculation	flow cytometry

Figure 2.2. The number counted of each cell type is multiplied by the shape factor. The morphological index for each RBC sample is then calculated by adding these products. The closer the morphological index is to 100, the closer the morphology of the sample is to the morphology of normal RBCs.

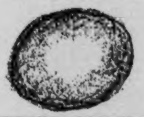
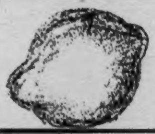
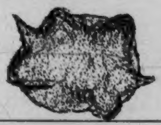
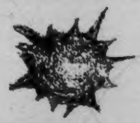
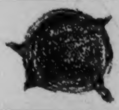
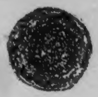
Description of RBC	Shape Factor	RBC Morphology
Smooth Disc	1.0	
Crenated Disc	0.8	
Crenated Discoid	0.6	
Crenated Spheroid	0.4	
Crenated Sphere	0.2	
Smooth Sphere	0.0	

Table 2.1: RBC quality measures in 2-3 d pre-freeze storage group. There was no statistically significant difference between the pre-freeze RBC quality results and the hypothermic controls for any of the assays performed.

RBC quality assay	pre-freeze (2-3 d storage)	post-thaw	24 h post-thaw	hypothermic controls (3-4 d storage)	p-value
hemolysis (%)	0.2 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.2 ± 0.1	0.962
MCV (fL)	95 ± 1	98 ± 4	98 ± 4	96 ± 4	0.933
MCH (pg)	32.0 ± 0.3	32.3 ± 1.5	32.3 ± 1.5	32.7 ± 1.4	0.859
MCHC (g/L)	338 ± 2	331 ± 8	330 ± 7	340 ± 7	0.890
ATP (μmol/g Hb)	3.4 ± 0.1	3.2 ± 0.4	3.2 ± 0.4	3.4 ± 0.8	0.891
extracellular K^+ (mM)	3.8 ± 0.2	3.7 ± 0.9	11.6 ± 2.0	5.7 ± 1.0	0.084
morphology index	96.1 ± 0.8	93.3 ± 3.2	88.0 ± 5.2	99.0 ± 4.3	0.588

Table 2.2: RBC quality measures in 13-14 d pre-freeze storage group. There was no statistically significant difference between the pre-freeze RBC quality results and the hypothermic controls for any of the assays performed.

RBC quality assay	**pre-freeze (13-14 d storage)**	**post-thaw**	**24 h post-thaw**	**hypothermic controls (14-15 d storage)**	**p-value**
hemolysis (%)	0.4 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	0.4 ± 0.2	0.974
MCV (fL)	96 ± 2	101 ± 3	101 ± 1	94 ± 6	0.778
MCH (pg)	31.3 ± 0.8	32.9 ± 1.2	33.0 ± 0.4	31.8 ± 2.3	0.872
MCHC (g/L)	329 ± 3	325 ± 6	325 ± 2	334 ± 6	0.692
ATP (μmol/g Hb)	3.6 ± 0.4	3.0 ± 0.4	2.9 ± 0.2	3.7 ± 0.7	0.747
extracellular K^+ (mM)	40.4 ± 10.7	2.5 ± 0.4	9.4 ± 0.5	27.6 ± 3.6	0.071
morphology index	90.9 ± 2.6	83.2 ± 4.8	77.2 ± 1.9	90.2 ± 2.5	0.862

Figure 2.3. A forward scatter (FSC) versus side scatter (SSC) plot with logarithmic scales was used to set a region around the RBC population (R1) and microvesicle population (R2). RBC- and microparticle-associated fluorescence intensity for annexin V and CD47 markers was then measured.

Figure 2.3.A: RBC positive control, where microvesiculation was induced with NEM reagent

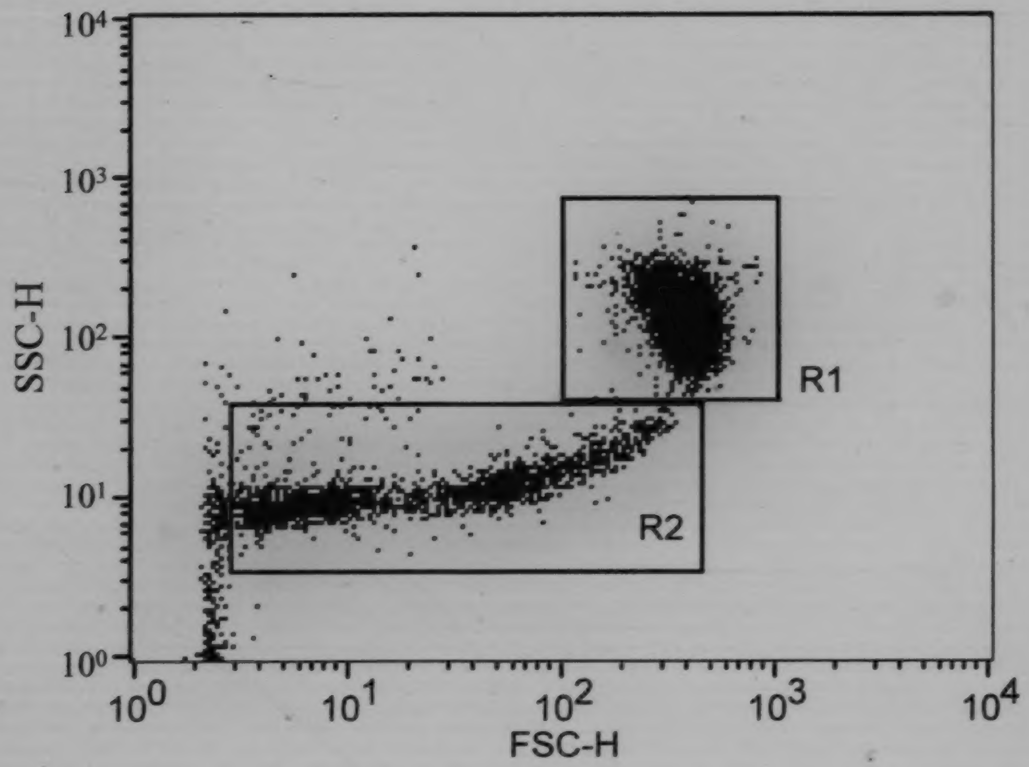


Figure 2.3.B: 400 nm standard latex beads (R2) with RBCs

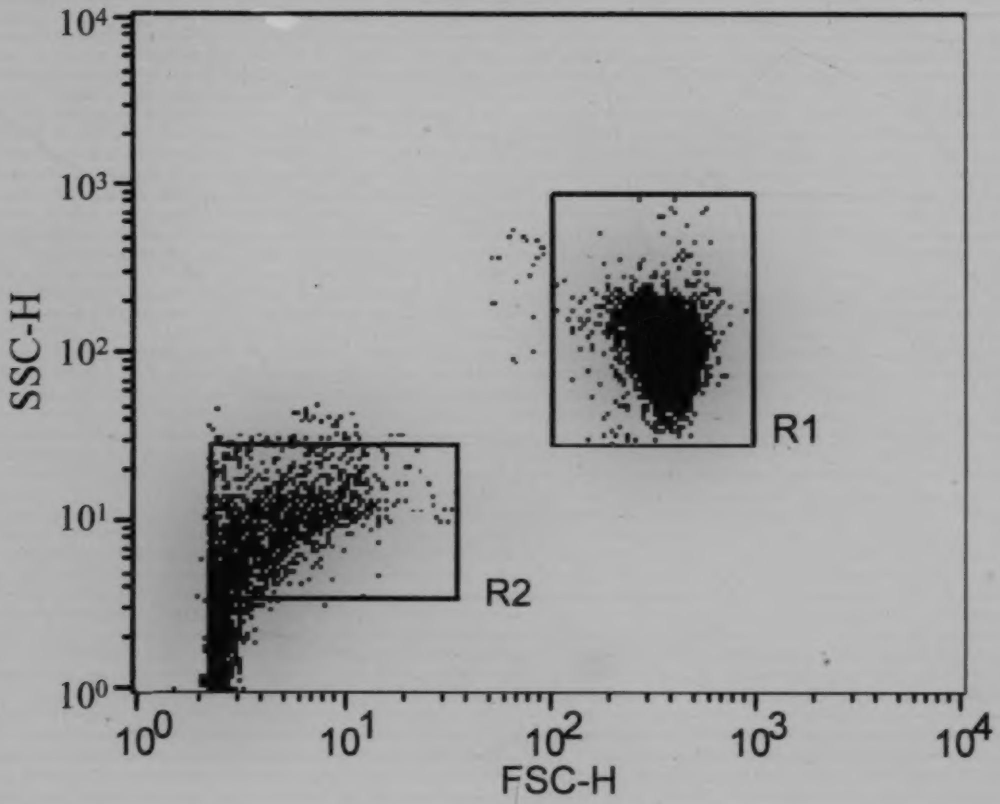


Figure 2.3.C: An example of a RBC sample 24 h post-thaw

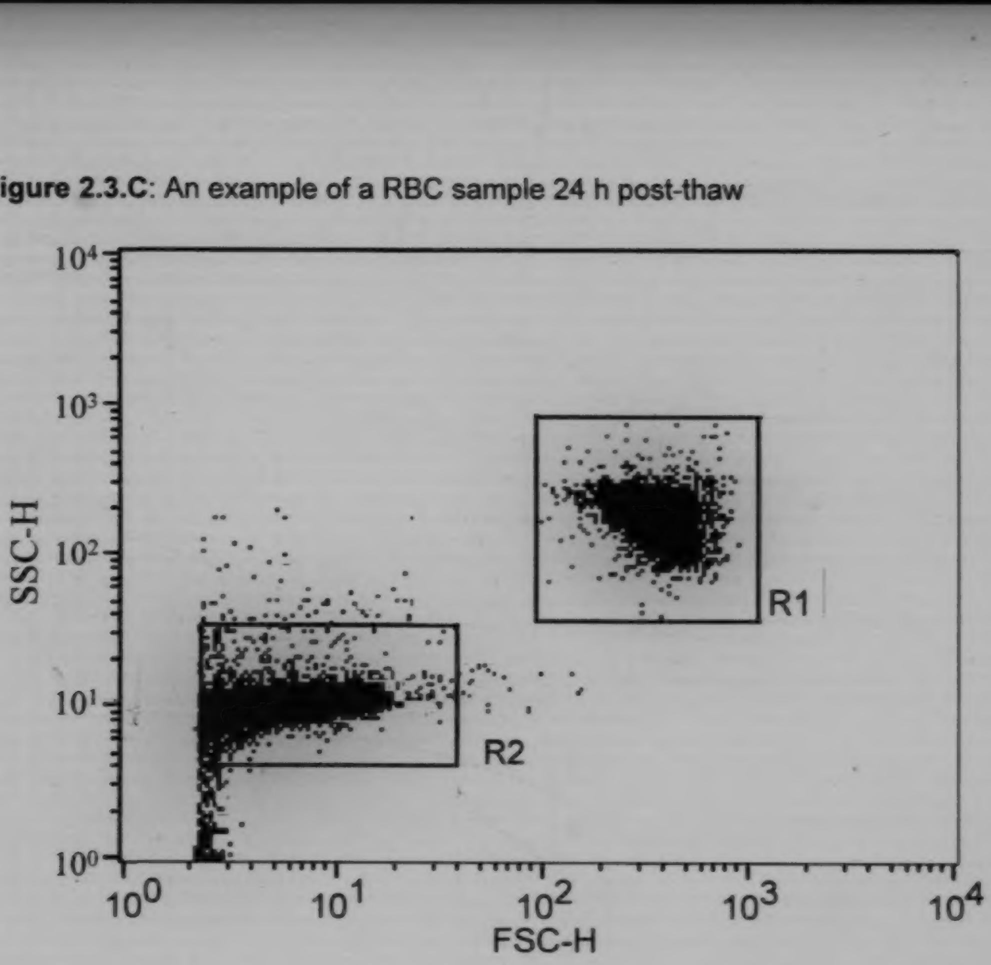


Figure 2.4. PS exposure of cryopreserved RBCs. After 24 h of post-thaw storage a significant increase in PS exposure was seen in the 13-14 d stored units as compared to the 2-3 d stored units (*p=0.016). In addition, following 24 h of post-thaw storage, PS exposure was significantly higher compared to pre-freeze levels (*p=0.021).

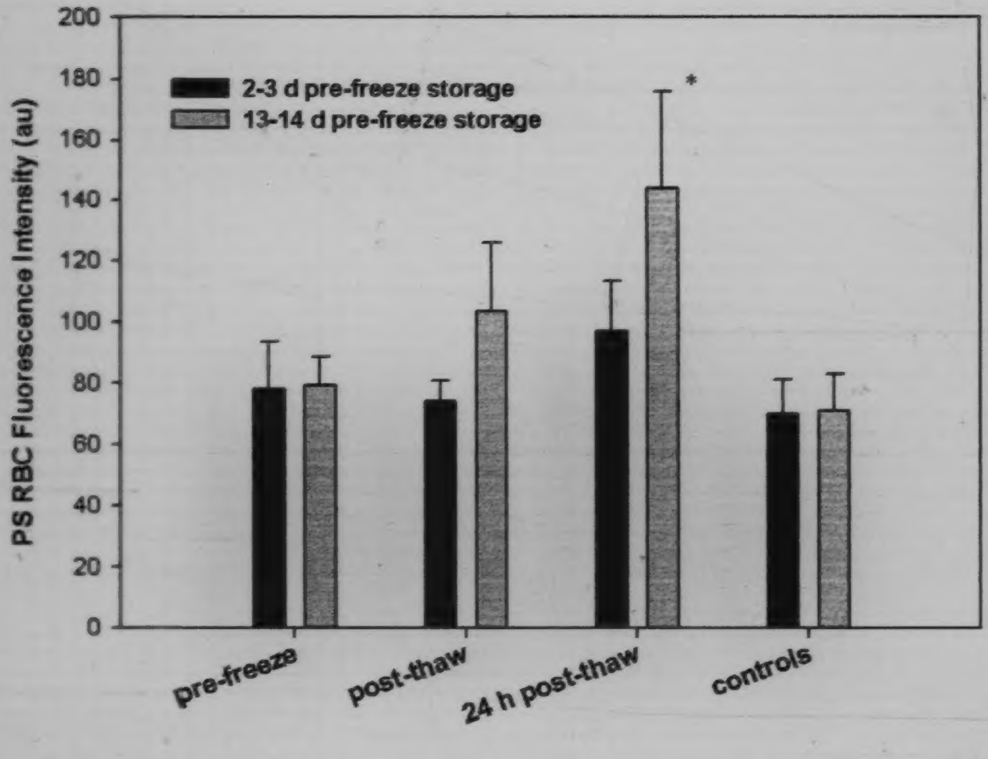


Figure 2.5. CD47 expression of cryopreserved RBCs.In both test groups, CD47 expression did not significantly change after cryopreservation or following the 24-h post-thaw period.

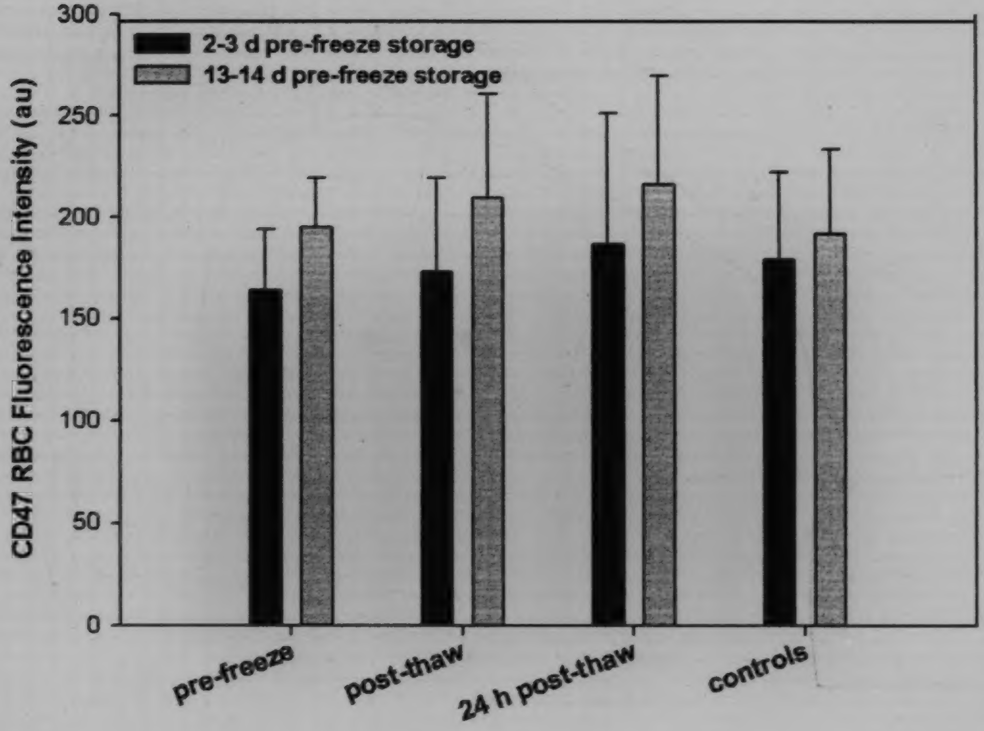


Figure 2.6. Microvesiculation of cryopreserved RBCs. While pre-freeze and post-thaw hypothermic storage resulted in microvesicle accumulation, the freezing process itself did not contribute to microvesiculation. Prolonged pre-freeze storage resulted in significant accumulation of microvesicles after 24 h of post-thaw hypothermic storage (p=0.017).

†Percent events are calculated by dividing the number of events in the microparticle gate by the sum of RBC and microparticle events.

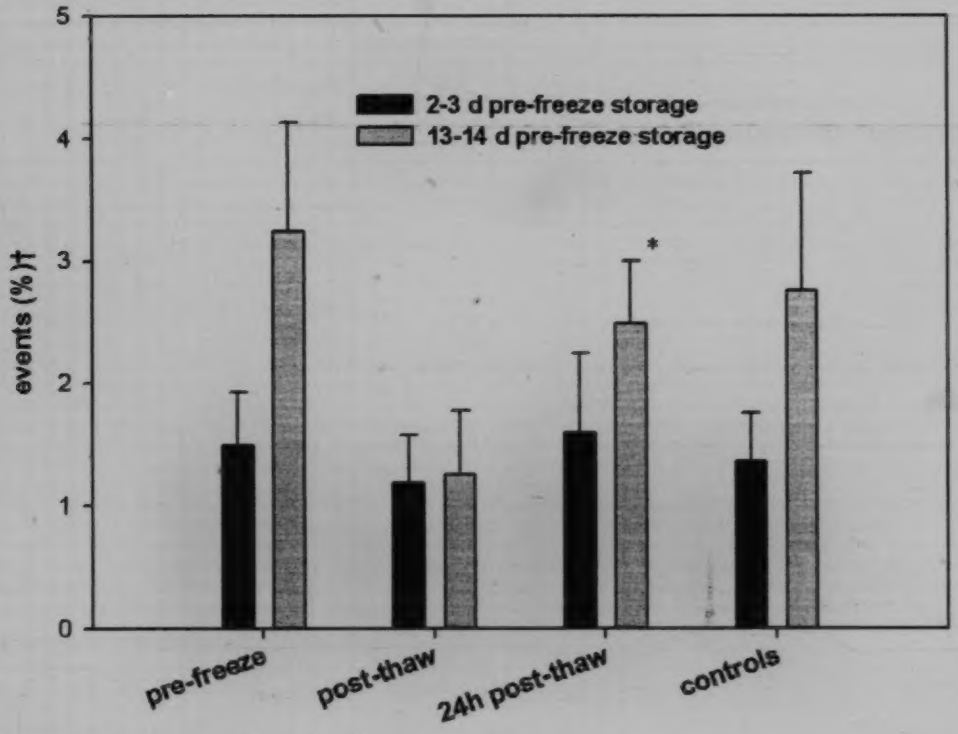


Figure 2.7. PS expression of RBC microparticles. After 24 h of post-thaw storage, a significant increase in PS fluorescence intensity per microparticle compared to pre-freeze values was seen in the both 2-3 d and 13-14 d stored units (*p<0.001).

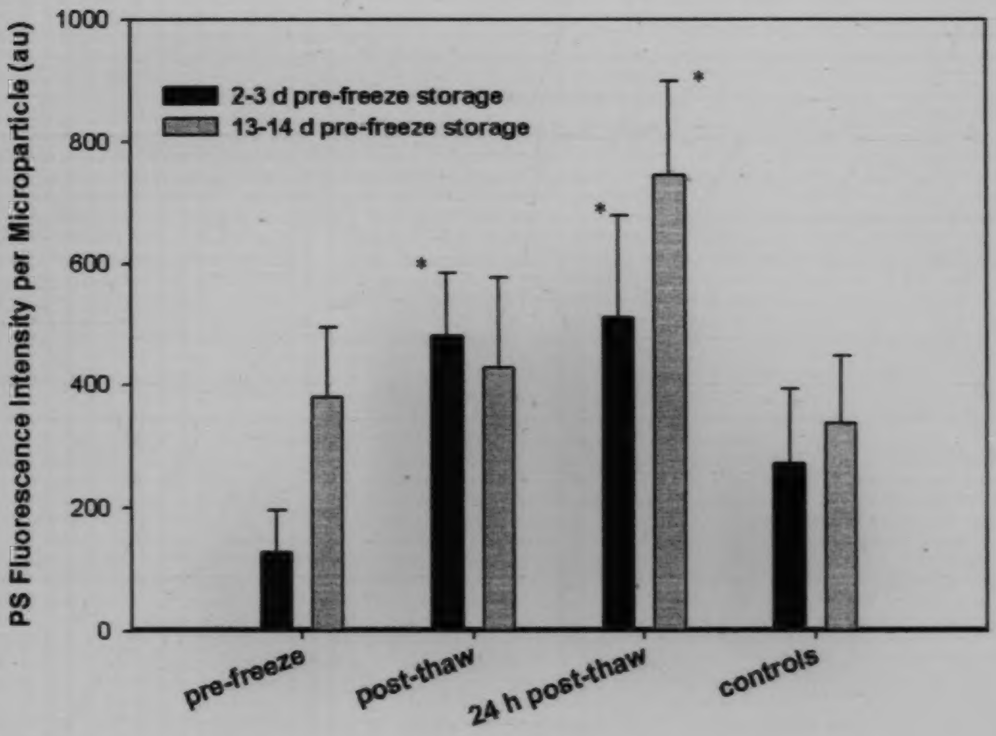


Figure 2.8. CD47 expression of RBC microparticles. There was no statistically significant difference in CD47 fluorescence intensity per microparticle between the test groups or between time points.

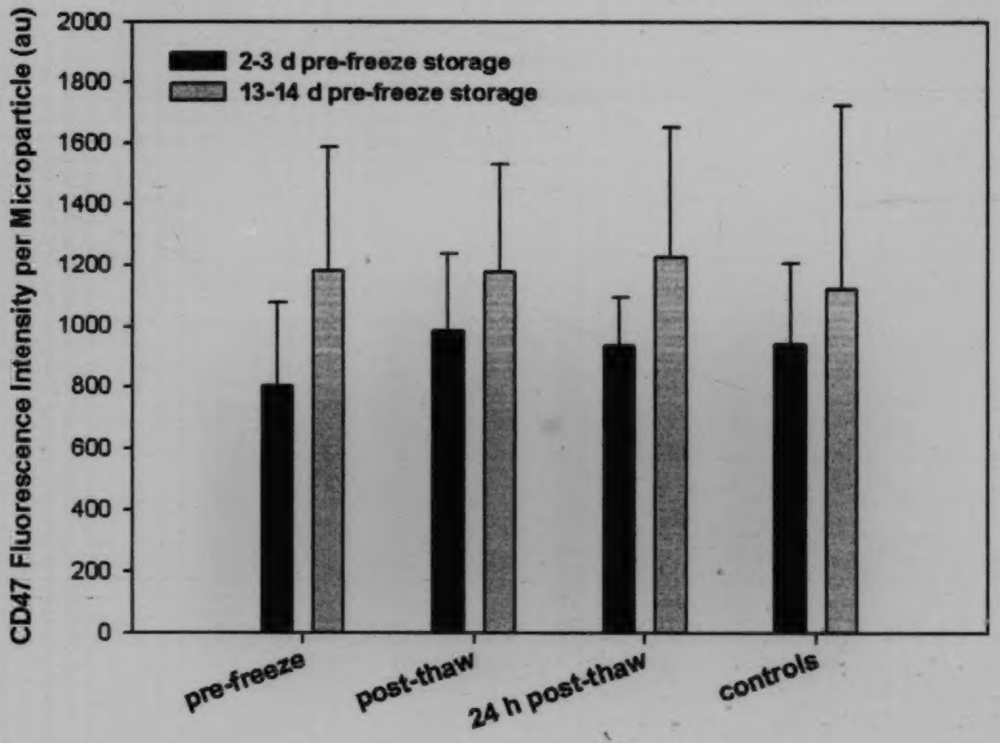
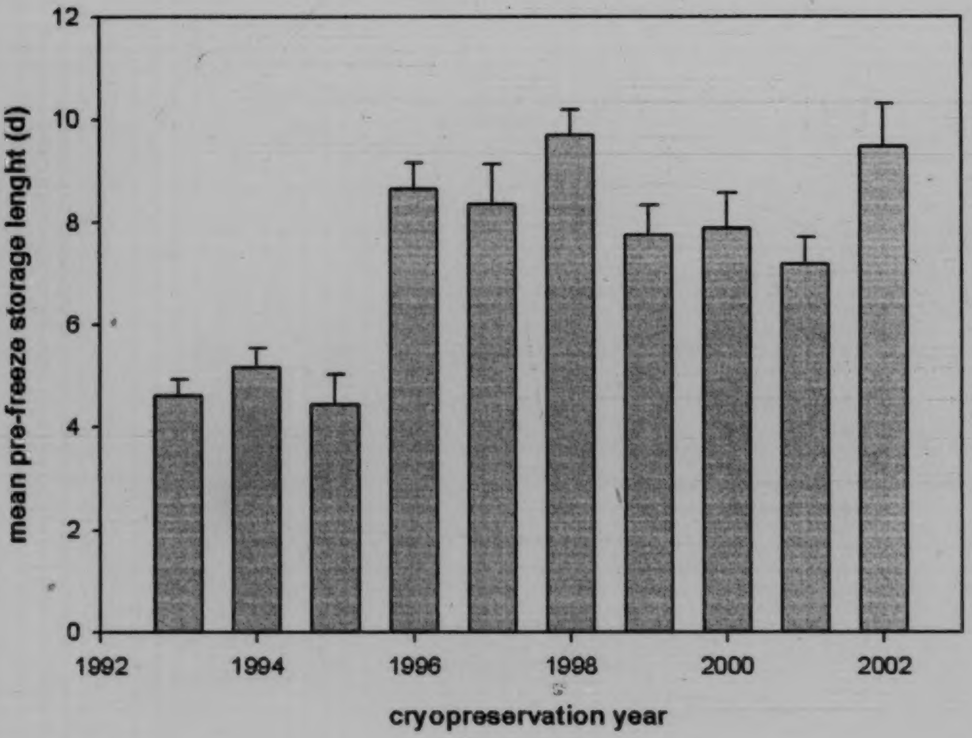


Figure 2.9. The average length of time packed RBC units are stored hypothermically prior to cryopreservation in CBS Edmonton Centre between 1993 and 2002 is substantially less than that permitted by existing standards (7.3 ± 2.0 d, range 1-40 d), but has continuously increased over the past 10 years (1993-1997 average 5.9 ± 1.8 d; 1997-2002 average 8.6 ± 0.9 d).



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Chapter 3*

Synthesis and Characterization of Trehalose-containing Liposomes for Applications in Biopreservation

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3.1. Introduction

Trehalose, a non-reducing glucose disaccharide found at high concentrations in many species of anhydrobiotic organisms, shows significant promise in protecting cellular viability and structural integrity during freezing and desiccation. Although a number of biochemical properties contributing to the exeptional ability of trehalose to stabilize biological structures under suboptimal conditions have been described,[1; 2; 3; 4; 5] the exact mechanism by which trehalose confers dessication and freeze tolerance has not been completely delineated. The potential applications of intracellular disaccharides in cell biopreservation is limited by the inability of mamalian cells to synthesize or actively accumulate such sugars as trehalose. Several experimental approaches have been developed for incorporating trehalose into mammalian cells, including introduction of genetically engineered mutant of a pore protein,[6; 7] thermal poration,[1; 8] genetic modification,[2; 9] microinjection,[10] $P2X_7$ receptor poration,[11] electroporation,[3] and permeabilization by femtosecond laser pulses.[12] As these techniques have been shown to have multiple technical and practical limitations,[5; 13] we are investigating the use of liposomes as a permeabilization strategy for the intracellular delivery of trehalose.

Liposomes are synthetic, microscopic, spherical vesicles composed of one or more concentric lipid bilayers enclosing an equal number of aqueous core compartments. They can form spontaneously when amphipatic molecules aggregate in aqueous media to form bilalyer membranes of closed structures with concentric lamellae.[14; 15; 16] The initial focus of liposome research was biochemical – liposomes were viewed as simple synthetic analogues of natural membranes, facilitating their use for studying cell membrane properties, such as structure and fluidity. Since liposomes synthesized from naturally-occurring phospholipids are biodegradable, nontoxic and nonimmunogenic, over the last couple of decades these vesicles have gained appreciation as a useful cell delivery vehicle for both water- and lipid-soluble membrane-impermeant biomaterials.[15; 17; 18; 19] For example, they are used in the pharmaceutical and medical industry as drug carriers and controlled release systems of anti-tumor, anti-microbial, and anti-inflammatory agents in cancer, gene, arthritis, metal chelation and hemophilia therapies.[16; 17; 18; 19; 20; 21; 22; 23; 24; 25] Another useful application of liposomes in these fields involve controlled delivery of diagnostic agents, such as radiopharmaceutical markers and signal carriers.[17; 18; 26] In immunology, antigens, interleukins and cytokines encapsulated in liposomes are used as immunoadjuvants for vaccine development and as immunomodulators.[16; 18; 27; 28] Many other scientific disciplines have benefited from the applications of liposomes, including food and cosmetic industry, chemistry, genetic engineering, toxicology and ecology.[29]

A similar trend can be seen in biopreservation research. Crowe *et al.* pioneered the use of liposomes as model membranes to study mechanisms by which trehalose protects cell membranes during lyophilization.[30; 31; 32] Other investigators focused on liposomes as tools to characterize cell behavior during freezing, thawing, and vitrification; ice propagation; phospholipid liquid crystalline-gel phase transition temperatures; as well as the cryoprotective action of permeating and nonpermeating additive solutions.[33; 34; 35; 36; 37; 38; 39; 40] Instead of treating liposomes as cell analogues, we are focusing on applications of liposomes as delivery tools. Therefore, the purpose of this study is to adapt existing techniques in liposomal research to synthesize and characterize trehalose-containing liposomes to be used for the intracellular delivery of this membrane impermeable stabilizing disaccharide. Multiple methods were applied in this study to qualitatively and quantitatively assess synthesized trehalose-containing liposomes, including size, concentration, morphology, population homogeneity, membrane composition, and trehalose encapsulation. Use of liposomes as delivery vesicles requires a consistent and well characterized product, which is essential for controlled, reproducible and effective delivery of protective intracellular disaccharides into mammalian cells for applications in biopreservation.

3.2. Materials and Methods

Experimental steps used for synthesis and characterization of trehalose-containing liposomes are illustrated in Figure 3.1.

3.2.1. Liposome Synthesis[*]

Multilamellar vesicles (MLVs) were prepared by the technique of Bangham *et al*,[14] as modified by Olson *et al.*[41] Dry lipids 1,2-dipalmitoyl-*sn*-glycero-3-

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[*] Protocols and training for liposome sythesis were kindly provided by Dr.Maria Gyongyossy-Issa and Iren Constantinescu at the University of British Columbia. The author would like to acknowledge Kirby Scott's help in adopting this protocol for synthesis of trehalose-containing liposomes.

phosphacholine (DPPC) (Sigma-Aldrich, St. Louis, MO) and cholesterol (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform (Alfa Aesar, Ward Hill, MA) and thoroughly mixed at a molar ratio of 7:3, resulting in a 25 mM final lipid solution. Chloroform solvent was evaporated from the lipid mixture using a dry nitrogen stream, leaving behind a thin film of dried lipid on the sides of a glass vial, which was then lyophilized for at least 12 hours, ensuring removal of residual solvent (Flexy-Dry Lyophilizer, FTS Systems, Stone Ridge, NY).

Dry lipids were hydrated with several buffers, according to Table 3.1. Hydrating buffers were warmed to 60 ºC before the addition to dry lipid film. Dispersion hydration method consisted of rigorous vortexing for approximately 3 min, warming the vortexed mixture in a 60 ºC waterbath to increase liposome membrane fluidity, then repeating 5 times. The suspension was then incubated on a shaker at 4 ºC overnight, to allow for further swelling of phospholipid-cholesterol films, resulting in formation of MLVs characteristically heterogeneous in size (1-10 μm in diameter), shape and number of lamellae per vesicle.[42; 43]

The hydrated MLV suspension was then subjected to a freeze-thaw protocol, involving 5 cycles of freezing the samples in liquid nitrogen, thawing in 60 ºC waterbath, and vigorous vortexing. Freeze-thaw protocol increases intralamellar liposome spaces and improves homogeneity of MLV suspension, lipid hydration and transmembrane solute distribution, resulting in a larger volume entrapped in the liposomal aqueous core.[42; 43]

MLVs were disrupted to generate a homogeneous population of large (~400 nm) unilamellar vesicles (LUVs) using a lipid extrusion technique.[29; 41]

The MLV suspension (4-8 mL) was introduced into the inlet chamber of an extruder (Lipex Extruder, Northern Lipids, Vancouver, BC) and forced through polycarbonate filters (25 mm diameter) with 400 nm pore size (Nuclepore Polycarbonate, Whatman, Newton, MA) under nitrogen pressure of 2000 kPa and temperature of approximately 60 °C. Extrusion process was then repeated 10 times to obtain homogeneous unilamellar vesicles with a diameter near the pore size of the polycarbonate filter.

The liposome suspension was then washed to remove hydrating solution not encapsulated inside the liposomes by repeated centrifugation (12800 x g, 15 min, 21 °C, 3 times) in saline buffers, according to Table 3.1. After the third wash, liposome preparations were stored under nitrogen gas to minimize phospholipid oxidation, and refrigerated (4-6 °C) for up to 5 weeks until required for experiments.[44]

3.2.2. Liposome Characterization

Synthesized liposomes were characterized using several techniques, as illustrated in Figure 3.1

3.2.2.1. Liposome Phosphate Content[*]

Phospholipid content of liposome preparations were specrophotometrically quantified using a Fiske-Subbarow colorimetric assay, that was adapted form the work of Barlett.[29; 45] This assay is based on the ability of perchloric acid to release phosphorus from phospholipids and oxidize it to inorganic phosphate when heated at 180-200 °C for 1.5 hours. Therefore, 10 µL of liposome sample was added to 700 µL of perchloric acid, while 10 µL of DPPC stock solution was used as a control. Addition of 7 mL ammonium molybdate solution (5.6 mM ammonium molybdate in 1 M sulphuric acid solution, Sigma-Aldrich, St. Louis, MO) converts the inorganic phosphate to phospho-molybdic acid, which is reduced to a blue colored phosphomolybdic acid complex upon addition of 900 µL Fiske-Subbarow reagent (1.4 M sodium bisulfite, 40 mM sodium sulfite, and 10 mM 1-amino-2-naphthol-4-sulphonic in distilled water) and heating in a boiling waterbath for 15 min. The absorbance of the blue-colored complex in the phosphate standards and LUV preparations were measured specrophotometrically at 815 nm (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). A phosphate assay standard curve was obtained by plotting the absorbence against the phosphate concentration of standards (0, 25, 50, 75, 100, 200 and 300 nm NaH_2PO_4), and further used to calculate the phospholipid content of liposome preparations, according to the following equation:

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[*] The author would like to acknowledge Iren Constantinescu for her help with Fiske-Subarrow phosphate assay and the Department of Department of Agricultural, Food and Nutritional Science (University of Alberta) for the permission to use their phosphoric acid fume hood.

$$total\ lipid\ concentration\,(mM) = \frac{C_{PO_4} \times V_S}{\%_{PO_4}} \times 100 \qquad \text{(Eq. 3.1)}$$

where C_{PO_4} = phosphate concentration (nM) from the absorbance standard curve

Vs = volume of liposome or DPPC stock solution in µL

$\%_{PO_4}$ = percent of phosphate in the sample (100 for the DPPC stock and 70 for liposome preparations)

In addition to liposome phospholipid content, the average phospholipid recovery was calculated by comparing the concentration of starting phospholipid stock solution (DPPC in chloroform) and the final phospholipid concentration of synthesized liposomes.

3.2.2.2. Liposome Size

The homogeneity and mean diameter of synthesized liposomes were determined using a Mastersizer 2000 Hydro SM system* (Malvern Instruments, Worcestershire, UK), which is based on laser light scattering principle.[46] Approximately 500 μL of liposome preparation was added to the unit dispersion tank filled with the appropriate external liposome buffer (300 or 400 mOsm HBS), and the pump stirrer was set at 1000 rpm. As liposomes are passed through a laser beam (wavelength 633 nm), light is scattered at an angle that is proportional to the particle size. Large particles will scatter light at narrow angles with high intensity, whereas small particles will scatter at wider angles with low intensity. As particle size decreases, the observed scattering angle increases logarithmically, which is measured by a series of photosensitive detectors. Polystyrene and silica 200, 400, and 800 nm sizing beads were used as controls (Bangs Laboratories Inc., Fishers, IN).

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* The Mastersizer system was kindly provided by Dr. Xu at the Department of Chemical and Materials Engineering (University of Alberta). Training on the instrument was provided by Jim Skwarok.

3.2.2.3. Liposome Morphology[*]

TEM was used to visually assess the morphology and size of the final LUV product. Final liposome solution was diluted 1/100 in appropriate external liposome buffer and one drop was placed in a perforated carbon film supported by 200-mesh copper electron microscope grids. The grid was then negatively stained with 2 % uranyl acetate, 1% trehalose solution.[47; 48] Excess solution was drawn off by touching a piece of wedged filter paper to the edge of the TEM grid, leaving a thin aqueous film on the grid surface, which was then air-dried. TEM investigations were conducted using a Philips/FEI Tecnai F30 electron microscope. Digital image acquisition was by a CCD camera, while the microscope was controlled through the Morgagni user interface and integrated image analysis software (AnalySIS® Software, Soft Imaging Systems, Lakewood, CO, USA). This system allows a resolution of 0.5 nm with magnifications up to 200,000 X. Electron micrographs were obtained at magnification between x5,600 and 18,000. Instrumental magnifications were calibrated using 400 nm latex standard beads (Bangs Laboratories Inc., Fishers, IN). In addition to visual assessment of synthesized liposomes, TEM image analysis program was used for independent verification of liposome size. The volume of the liposome aqueous core can be then calculated based on the spherical vesicle shape, measured particle size and an assumed lipid bilayer thickness of 5 nm.[15]

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[*] Training and assistance with TEM was kindly provided by Rakesh Bhatnagar at the Advanced Microscopy Facility (University of Alberta).

3.2.2.4. Liposome Number

The number of liposomes was determined theoretically. Previously, Lasic[16] published a graph of the liposome radius as a function of the number of phospholipid molecules. We chose 10 points on this curve, inverted the data and then fit those to a 10-degree polynomial[*] (number of phospholipid molecules as a function of liposome radius, Figure 3.2). This polynomial allowed us to predict the number of phospholipid molecules in a synthesized liposome of a known size, assuming that the surface area of a phospholipid polar head is 0.7 nm^2 in the inner and outer lipid monolayer. The curve fit to the data was evaluated using the Sum of Squares due to Error (SSE) statistical calculation (Microsoft Excel 2005, Microsoft, Redmond, WA). The number of phospholipid molecules in a synthesized liposome was then related to the number of phospholipid molecules per measured mM lipid, as described by Papahadjopolous *et al.*,[49] which allowed us to determine the theoretical number of vesicles in a volume of liposome suspension with known mean vesicle size and phospholipid concentration.

3.2.2.5. Intra-liposomal Trehalose Content

Trehalose-containing liposomes resuspended in HBS buffer (1 mM lipid concentration) were lysed using 1 % (wt/vol) Triton-X 100 detergent (Sigma-Aldrich, St. Louis, MO). The trehalose concentration of the liposome extract was then measured either spectrophotometrically, using a commercially-available assay (Megazyme International Ireland Ltd., Wicklow, Ireland), or using a normal-phase partition HPLC, containing a Varian 9010 solvent delivery system (Palo Alto, CA), a Hewlett Packard 1050 autosampler (Hanover St. Palo Alto, CA), a Varian Galaxie Data System software (Grenoble, France), and an evaporative light scattering detector (ELSD Alltech 500, Waukegan, IL).

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[*] The author would like to acknowledge Juan Irizar's assistance with curve fitting and polynomial equations.

The spectrophotometric assay involved the hydrolysis of trehalose into glucose by trehalase, followed by the colorimetric conversion of glucose to gluconate-6-phosphate. The amount of NADPH formed in this reaction is stoichiometric with the trehalose concentration in the sample, and was spectrophotometrically measured by the increase in absorbance at 340 nm (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). The concentration of trehalose may be calculated directly from the absorbance of the solution using the following equation:

$$trehalose(g/L) = \frac{\Delta A \times V \times MW}{\varepsilon_{340} \times v \times d \times 2} \qquad \text{(Eq. 3.2)}$$

where ΔA = (absorbance of the sample solution at 340 nm) – (absorbance of the blank solution at 340 nm)

V = total volume of the sample

MW = molecular weight of trehalose (g/mol)

ε = extinction coefficient of NADH (6300 @ 340nm)

v = sample volume used (mL)

d = light path (cm)

2 = two molecules of D-glucose released from each molecule of trehalose hydrolysed

The spectrophotometric assay was further characterized in terms of sensitivity, specificity, linearity, cost and safety. Trehalose solutions of various concentrations (0.001 mM to 500 mM) were used to verify the sensitivity and linearity of both the spectrophotometric and HPLC trehalose measurement methods. The specificity of the spectrophotometric method was determined by investigating possible interference from D-glucose in the sample. Liposomal lysates were split into two aliquots. Trehalose concentration was directly measured in the first aliquot, while in the second aliquot, free D-glucose was reduced to alcohol by alkaline sodium borohydride according to the Megazyme™ product insert instructions, prior to trehalose measurement.

During the HPLC analysis[*], supernatants containing liposomal aqueous constituents (20 µL) were added to the mobile phase containing gradient of 10 – 90 % HPLC-grade water-acetronitrile solution, and run through a Supelcosil LC-NH_2 HPLC amino-polyhydroxyethyl analytical column (4.6 mm x 25 cm, 5 µm particle size, 120 Å pore size, Supelco, Bellefonte, PA). The chromatographic separation was carried out in 36 min at a flow rate of 0.9-1.5 mL/min at room temperature (~22 °C). Nitrogen was used as the ELSD nebulizer gas (350 kPa, 4.0 L/min), at a temperature of 130 °C. The total trehalose concentration was calculated from the chromatogram peak areas of standard trehalose solutions of known concentration.

Unless indicated otherwise, the data points represent the mean values from three experiments with error bars denoting the standard deviation of the mean. Standard curve fits to the data were calculated using the linear regression calculation (Microsoft Excel 2005, Microsoft, Redmond, WA). By taking into consideration the calculated values for the liposome core volume and the number of liposomes in solution, the measured trehalose concentration was expressed in terms of the intra-liposomal trehalose content, as explained in the (Appendix A.1). Intra-liposomal trehalose encapsulation was then calculated by comparing the intra-liposomal trehalose content to the trehalose concentration in the hydrating buffer solution (Appendix A.1).

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[*] Training and assistance with HPLC was kindly provided by Gary Sedgwick at the Department of Agricultural, Food and Nutritional Science (University of Alberta).

3.3. Results

The phosphate assay was used to determine the phospholipid content of the different liposome preparations (Figure 3.3). These values were then used to determine the total lipid concentration of the samples. The mean total lipid concentration of the liposome preparations was 22.4 ± 1.9 mM, indicating on average recovery of 89.7 ± 7.2 % of lipid concentration in the phospholipid solution in the first step of liposome synthesis (25 mM of DPPC and cholesterol in chloroform).

Quasi-electric laser light scattering showed that 95 % of the lipid in the liposome suspension was in the form of particles ranging from 224-578 nm in size. Repeated extrusion resulted in liposome vesicles with a mean diameter of 401 ± 88 nm (Figure 3.4). The size analysis also revealed a unimodal Gaussian population distribution of liposomes, suggesting homogeneity of synthesized trehalose-containing liposomes that is independent of liposome concentration inside liposomes. The average sizes of the polystyrene and silica standard sizing beads used as controls were 142 ± 36 nm for the 200 nm beads, 328 ± 49 nm for the 400 nm beads and 778 ± 84 nm for the 800 nm beads (Bangs Laboratories Inc., Fishers, IN).

TEM demonstrated successful formation of trehalose-containing liposomes with a characteristic spherical shape (Figure 3.5). As repeated extrusion was previously shown to result in a unilamellar liposome product,[41; 43] we assumed a lipid bilayer thickness of 5 nm.[15] Using this value together with the measured mean liposome size and visualized spherical shape allowed us to calculate the volume of the liposomal aqueous core to be 0.03 fL.

Due to the small liposome size, obtaining an accurate liposomal count in a solution has been one of the major challenges of liposomal research.[50] Therefore, we determined this value theoretically. Previously published data relating the number of phospholipid molecules in a single vesicle as a function of vesicle radius[51] was fit to a 10-degree polynomial equation to calculate the number of phospholipid molecules in a trehalose-containing liposome (with a radius of 200.5 nm) and was found to be $0.96\text{x}10^4$ (Figure 3.2). The calculated value for the goodness of fit statistic SSE was close to zero, indicating that the calculated model had a small random error component, and therefore, matched the original data set well (SSE = $1.048\text{x}10^{-17}$). According to Papahadjopoulos *et al.*,[49] 1 mM phospholipid represents $6\text{x}10^{17}$ phospholipid molecules, which allowed us to relate liposome preparation of measured lipid concentration to the calculated number of phospholipid molecules in a trehalose-containing liposome, and therefore, liposome count (i.e. 1 mM lipid liposome preparation = 6.32×10^{13} liposomes).

Determining the number of liposome vesicles in a solution was essential in evaluating intra-liposomal trehalose encapsulation efficiency. Trehalose concentrations in a range 0.001 mM - 500 mM were used to verify the sensitivity and linearity of both the spectrophotometric and HPLC trehalose measurement methods. First, the sensitivity of the spectrophotometric trehalose measurement assay was determined to be 7 μM, while the linearity was between 7 μM - 3 mM trehalose (Figure 3.6A). The level of detection of the HPLC-ELSD trehalose measurement method was 0.25 mM, with a linear response between 0.25 and 400 mM trehalose (Figure 3.6B). The trehalose concentration of the 1 mM lipid liposomal lysate was spectrophotometrically determined to be 364 ± 38 μM (Figure 3.7), and was confirmed with HPLC measurements (397 ± 53 μM, Figure 3.8) for 200 mM trehalose liposomes. For 300 mM trehalose liposomes, the spectrophotometrically measured liposomal lysate concentration was 535 ± 42 μM (Figure 6A), and the HPLC-measured value was 515 ± 21 μM (Figure 3.8). Finally, for 400 mM trehalose liposomes, the measured trehalose lysate concentrations were 719 ± 34 μM and 800 ± 16 μM (spectrophotometry and HPLC, respectively, Figures 3.7 and 3.8). There was no interference from D-glucose on the trehalose spectrophotometric measurements, as the total trehalose concentrations in the samples treated with sodium borohydride were similar to results obtained when trehalose concentration was directly measured in the liposomal lysate (369 ± 29 μM vs. 364 ± 38 μM, respectively, $p<0.001$). No trehalose was detected in the liposomal supernatants prior to lysis, indicating that the measured trehalose is intra-liposomal (Figure 3.9). In addition, there was no trehalose detected in the lysates of the control liposomes by either spectrophotometric or HPLC methods (data not shown). The spectrophotometrically measured trehalose concentration in the liposomal lysate was expressed as an intra-liposomal trehalose content of 181.3 ± 11.2 mM (Appendix A1). As the initial trehalose concentration in the hydrating buffer solution during liposome synthesis was 200 mM, the intra-liposomal trehalose content of 181.3 mM was expressed as an intra-liposomal trehalose encapsulation efficiency of 90.7 ± 5.6 %. This value was in agreement with the 300 mM and 400 mM trehalose liposomes (91.1 ± 8.2 % and 102.1 ± 9.4 %, respectively). The example of calculations used to translate trehalose concentrations measured in liposomal lysate by either HPLC or spectrophotometry to intraliposomal trehalose concentraton and intra-liposomal trehalose encapsulation efficiency is shown in the Appendix A.1.

3.4. Discussion

Liposomes can be custom-designed to act as delivery vesicles conveying a wide variety of bioactive substances into cells. This property has facilitated liposome use in many disciplines, including medical, biological and pharmaceutical sciences. As liposome properties and behavior mostly depend on the method of production, a synthesis protocol should be adopted according to the requirements of liposome intended use.[50] We have chosen to synthesize trehalose-containing large unilamellar vesicles (LUVs) via an extrusion technique. Due to their large size, controlled lamellarity and population homogeneity, these vesicles exhibit a much higher encapsulation efficiency in terms of volume aqueous volume per mole of lipid, as compared to two other major liposome types, multilamellar vesicles and small unilamellar vesicles (SUVs). Therefore, LUVs have significant advantages for packing and delivering encapsulated compounds into cells. [19; 41; 42; 44] In addition, compared to other LUV synthesis techniques, extrusion has the benefit of being a simple, reproducible method that neither introduces impurities into the vesicles, nor induces phospholipid breakdown, making it possible to produce large volumes of homogeneous liposomes at high concentrations, and with a minimal dilution.[41; 44; 50]

In the presence of an aqueous phase, the arrangement of anhydrous phospholipids into hydrated lipid bilayer vesicles, or liposomes, is spontaneous, suggesting that the encapsulation of water soluble molecules into the liposomes is inherent.[44] However, the successful encapsulation of target biomolecules into the liposomal aqueous core is not a trivial process, as it depends on multiple variables, including the liposomal synthesis method, the relative amounts of lipid and solute in the preparation, liposome size and lamellarity, as well as the intrinsic characteristics of the hydrophilic compounds to be incorporated in the liposomal core, such as size, polarity, and charge.[44; 52] Since we are synthesizing liposomes to be used as delivery vesicles for biopreservation purposes, characterizing liposome encapsulation efficiency by quantifying intraliposomal trehalose concentration is an essential step in this approach. In addition, as one of the key determinants of the biostabilizing efficiency of trehalose is the amount accumulated intracellularly, determining liposomal trehalose encapsulation efficiency is critical in evaluating liposomal delivery.

The main challenge in evaluating trehalose encapsulation efficiency of liposomal preparations was not the measurement of intraliposomal trehalose content of liposomal lysates, but relating this value to specific properties of synthesized liposomes, such as volume and number. Translating measured trehalose concentrations in a liposome lysate into a liposomal encapsulation efficiency value required extensive characterization of the liposome product. For example, in order to accurately calculate the volume of the synthesized vesicles, we had to experimentally determine the mean liposome diameter by a dynamic light scattering method and visually confirm spherical vesicle shape by TEM. Although the lamellarity of liposomes was not directly assessed, the preparation method involving freeze-thaw cycles and multiple extrusions is highly conducive to the production of unilamellar liposomes, which allowed us to include the lipid bilayer thickness for a more precise liposome volume calculation. [41; 43] It has been demonstrated by more than 40 researchers that when liposomes are repeatedly passed through small pore polycarbonate membranes under high pressure, the average diameter of the liposomes becomes progressively smaller as layers of lipid bilayers are removed from the liposome structure, reaching unilamellarity and the size of the membrane pores after 5-10 extrusion passes.[53; 54; 55]. Numerous techniques have been used to demonstrate unilamellarity of liposomes synthesized by the high pressure extrusion method, including freeze-fracture electron microscopy[56; 57; 58]; small angle X-ray scattering[56; 57]; binding of radiolabelled ions [43; 50], and P-NMR[53; 58; 59]. As qualitative and quantitative indicators of liposome unilamellarity determined by these techniques are in agreement, further establishing that the high pressure extrusion liposome synthesis method results in a unilamellar liposome product was beyond the scope of this study.

In addition to lipid volume, the liposome concentration in terms of count per volume of liposome suspension was theoretically determined. When the size of synthesized liposomes is in the submicron range, experimentally measuring the liposome count becomes difficult.[29; 50] More traditional methods of particle counting, such as the Coulter method for measuring electrical impedance, or flow cytometry for measuring the optical properties of particles, are primarily designed for the analysis of mammalian cells in the micron range. Therefore, 400 nm liposomes are at the limit of detection for these instruments, making it difficult to distinguish liposome particles from background noise. TEM quantification is possible, but is tedious and difficult due to negative staining artefacts.[41; 50] The traditional approach in liposome characterization in microencapsulation research is to determine vesicle size distribution, entrapped volume per lipid weight, and total phospholipid concentration.[29; 54; 55; 56; 60] By employing a theoretical approach, we were able to take liposome characterization one step further, and calculate the actual liposome count per mL solution. Therefore, in addition to using the phosphate assay to account for sythesized liposomes by determining phospholipid recovery, we were able to relate this value to the number of phospholipid molecules per measured mM lipid. Recent advances in optical flow cytometry could overcome the limitations of conventional flow cytometry regarding the analysis of nanoparticles.[61] For example, it would be beneficial to apply a nanometric particle enuminator for detection of fluorescently labelled viruses to liposomal research, such as one that was developed by Ferris *et al.* to accurately detect single nanoparticles with a diameter as small as 26 nm.[62] As we have limited access to such instrumentation, we relied on a theoretical model.

The main advantage of our theoretical approach in determining liposome count is simplicity. Our model can be easily applied to calculate the liposome count per volume of liposome suspension regardless of the liposome composition or application, as long as vesicle size and phospholipid content of the liposome preparation has been experimentally determined. However, our approach to determining liposome count is based on several assumptions and estimations that need to be recognized for proper application. First, unilamellarity of the liposome vesicles, and therefore, the bilayer nature of the liposome membrane is assumed. As liposomal lamellarity is determined by the method of production,[44; 50; 54; 63] our modelling should be reserved for the production of LUVs using a high-pressure extrusion method. Second, the value used for the surface area of a phospholipid polar head-group in the inner and outer lipid monolayer, 0.7 nm^2, was chosen as a commonly used average value for any phospholipid.[16] However, this value will depends on several factors, including the phospholipid type, hydrocarbon chain length, temperature, membrane hydration state, and the presence of other lipids in the bilayer.[64] Also, it is important to note that our theoretical model can only be applied for liposome vesicles 0.2 – 1.5 µm in diameter. The accuracy of the calculated values is confirmed by the high trehalose intraliposomal encapsulation efficiency values, which are in close agreement with the past reports evaluating trapping efficiencies of liposomes produced by high pressure extrusion method.[29; 41; 42; 43] Therefore, although the theoretical approach for obtaining liposome count is indirect and encompasses several assumptions, it provides researchers with a valuable tool for future liposome applications, such as determining optimum dosage conditions and delivery efficiency.

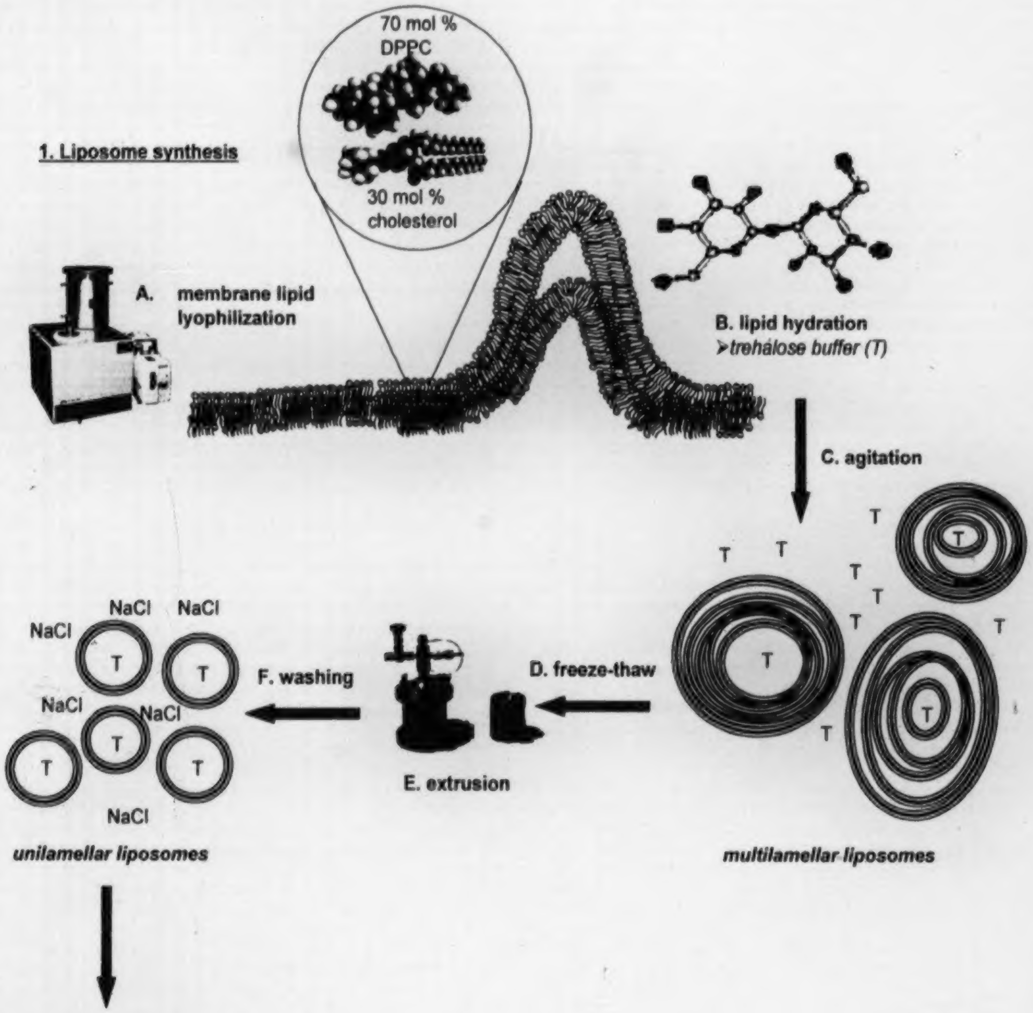
Since one of the key determinants of trehalose stabilization is the amount of trehalose that is accumulated intracellularly, quantifying trehalose concentration has become a critical step in many biopreservation approaches. Therefore, it is important to address the advantages and disadvantages of various trehalose measurement methods. Currently, there are several techniques used to determine trehalose concentration, including HPLC,[65; 66] gas chromatography-mass spectroscopy (GC-MS),[7] and spectrophotometric assays (anthrone reaction[1; 67; 68] and glucose-oxidase kit[69]). In this study, we used a commercially-available Megazyme spectrophotometric method to measure the trehalose content of the liposomal aqueous core. The sensitivity, specificity, cost, and ease of use make this method an attractive alternative to the approaches that have been used in past. For example, GC-MS is a very sensitive technique for the measurement of trehalose in biological samples, but it requires trehalose derivatization processes, such as trimethylsilylanization, which is time-consuming and involves the handling of carcinogenic reagents.[7; 65] There is no need for sample derivatization in the spectrophotometric Megazyme method, nor do the reagents require any special safety measures. Although the HPLC-ELSD method is linear over a broader range of trehalose concentrations,[65; 66] its sensitivity is lower than the Megazyme method (Figure 3.6). Also, it is important to note that both GC-MS and HPLC require access to expensive instrumentation and components, including columns, detectors, and computer software, as well as extensive technical expertise to run samples and analyze results, translating into a higher cost. In contrast, a spectrophotometer is the only required instrument for the Megazyme method. Unlike the Megazyme assay, the specificity of the anthrone method is quite low, as the results obtained represent the total saccharide content of the sample rather than the concentration of the specific sugar in question.[1; 67; 70] Moreover, this method gives misleading results at low trehalose concentrations.[71] Among the mentioned alternative methods for trehalose measurement, the glucose-oxidase spectrophotometric method is most comparable to the Megazyme method in principle and use. In contrast to the Megazyme kit, the disadvantages of the glucose-oxidase method are that the trehalase enzyme and glucose-oxidase assay kit must be purchased separately from two different suppliers, and that biological samples containing erythrocytes may interfere with absorbance measurements, due to the presence of hemoglobin, which also absorbs at 540 nm. However, a significant disadvantage of the Megazyme method is its linear range, with the upper limit of only 3 mM. Samples containing high trehalose concentration should be diluted sufficiently to yield a trehalose concentration in the linear range.

This chapter described how existing techniques in the liposomal research can be adapted to synthesize and characterize trehalose-containing liposomes. It has provided accurate measurements of liposome size, morphology, phosphate content, and intra-liposomal trehalose encapsulation efficiency. Therefore, it has set a solid stage for further investigation on applications of liposomes in the field of biopreservation.

3.5. Conclusion

Although the disaccharide trehalose was first associated with anhydrobiosis and biostabilization almost 50 years ago,[72] its impermeability through cell membranes has greatly hindered its application in the biopreservation field. Liposomal delivery could circumvent many difficulties associated with the previously mentioned permeabilization techniques - liposomes are nontoxic and nonimmunogenic, and their delivery would not require specialized equipment or extensive technical expertise. This chapter describes synthesis and characterization of trehalose-containing liposomes as the first step in establishing liposomes as delivery system in the biopreservation field. Liposome size, morphology, phosphate content, and high intra-liposomal trehalose encapsulation efficiency suggest the successful adaptation of techniques in liposomal research for the synthesis of trehalose-containing liposomes for applications in the field of biopreservation. Future chapters will address the interactions of trehalose-containing liposomes with human red blood cells, as well as the potential use of liposomes as trehalose delivery vesicles and cryoprotective agents.

Figure 3.1. Experimental steps for synthesis and characterization of trehalose-containing liposomes.


1. Liposome synthesis
70 mol % DPPC
30 mol % cholesterol
A. membrane lipid lyophilization
B. lipid hydration
➢*trehalose buffer (T)*
C. agitation
T
multilamellar liposomes
D. freeze-thaw
E. extrusion
F. washing
NaCl
unilamellar liposomes

2. Liposome characterization

A. lipid content
  - *spectrophotometry*

B. liposome size
  - *Mastersizer*

C. liposome morphology
  - *TEM*

D. liposome number
  - *calculated value*

E. intra-liposomal trehalose content
  - *Spectrophotometry, HPLC*

Table 3.1. Hydrating and external buffers used in liposome synthesis

Liposome Preparation	Hydrating Liposome Buffer Composition	External Liposome Buffer Composition
200 mM trehalose	200 mM trehalose, 40 mM KCl, 3.3 mM NaCl, 1.7 mM glucose, 6.7 mM HEPES (pH 7.4, 293 mOsm)	140 mM NaCl, 20 mM HEPES (pH 7.4, 298 mOsm)
300 mM trehalose	300 mM trehalose, 10 mM HEPES (pH 7.4, 306 mOsm)	
HBSI	120 mM KCl, 10 mM NaCl, 5 mM glucose, 20 mM HEPES (pH 7.4, 287 mOsm)	
400 mM trehalose	390 mM trehalose, 10 mM HEPES (pH 7.4, 398 mOsm)	190 mM NaCl, 20 mM HEPES (pH 7.4, 394 mOsm)

Figure 3.2. Previously published data relating the number of phospholipid molecules in a single vesicle as a function of vesicle radius [51] was fitted to a 10-degree polynomial equation to determine the number of phospholipid molecules in a liposome vesicle of known size $y=123-133.265x + 456.907x^2 - 354.103x^3 + 128.148x^4 - 21.708x^5 + 0.786049x^6 + 0.271013x^7 - 0.0381839x^8 + 0.00153603x^9$

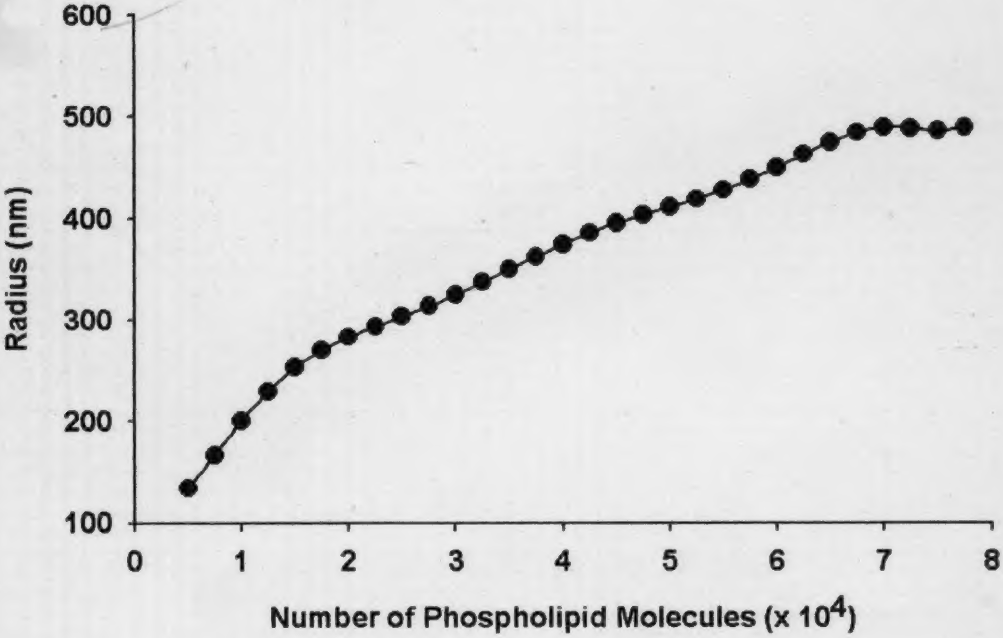


Figure 3.3. Spectrophotometric assay of the phosphate content was used to determine the total lipid concentration of the different liposome preparations, shown as triangles.

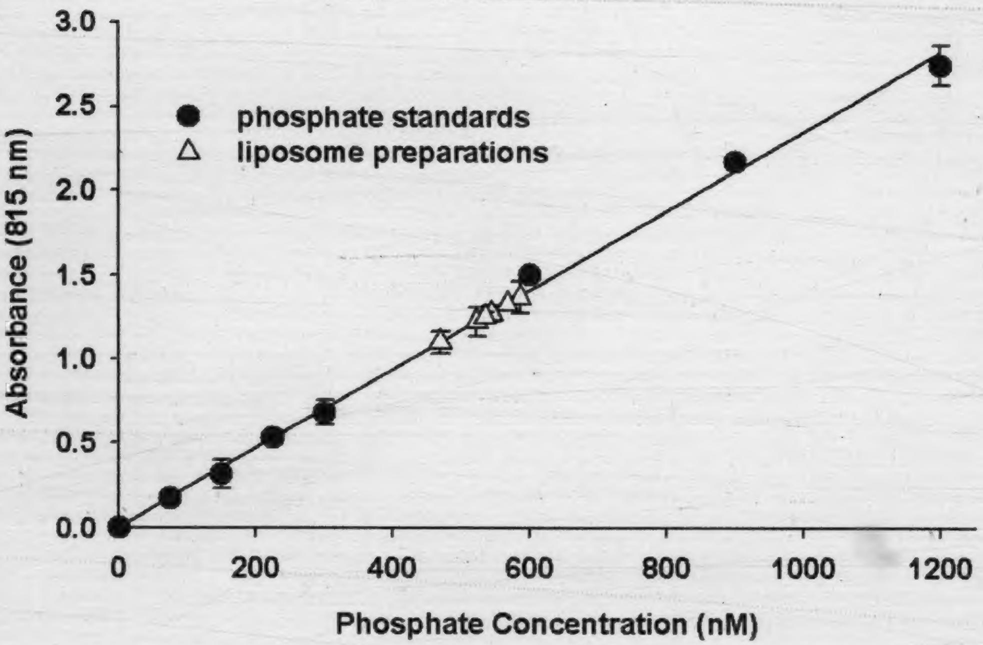


Figure 3.4. Repeated extrusion resulted in a unimodal, normal liposomal distribution, suggesting homogeneity of synthesized trehalose-containing liposomes that is independent of liposome concentration inside liposomes.

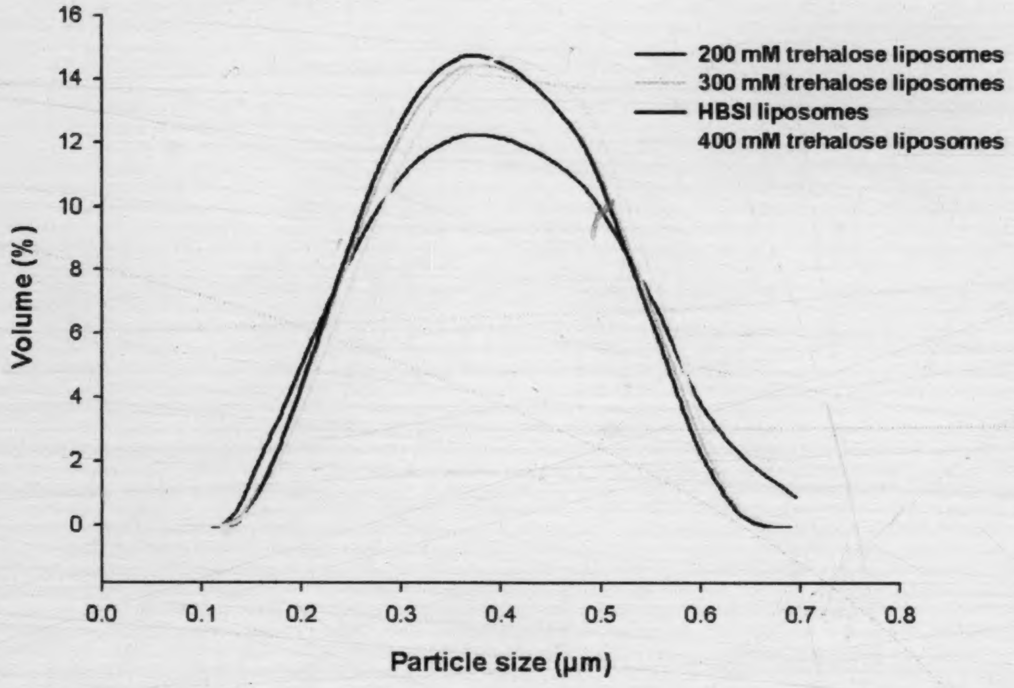


Figure 3.5. Standard latex beads (400 nm, top left picture) and synthesized liposomes were visualized by TEM.

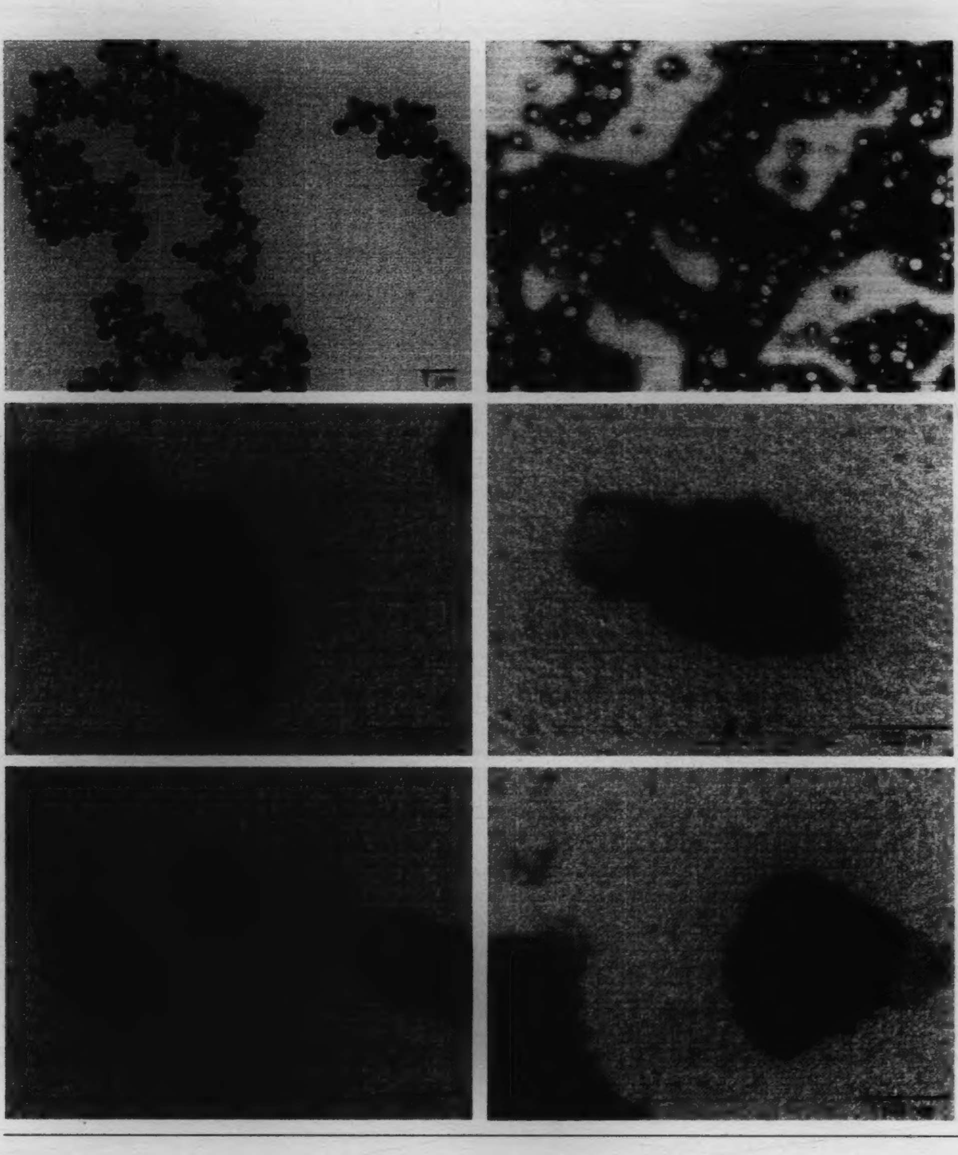


Figure 3.6. Sensitivity and linearity of spectrophotometric (Fig. A) and HPLC-ELSD trehalose measurement methods (Fig B).

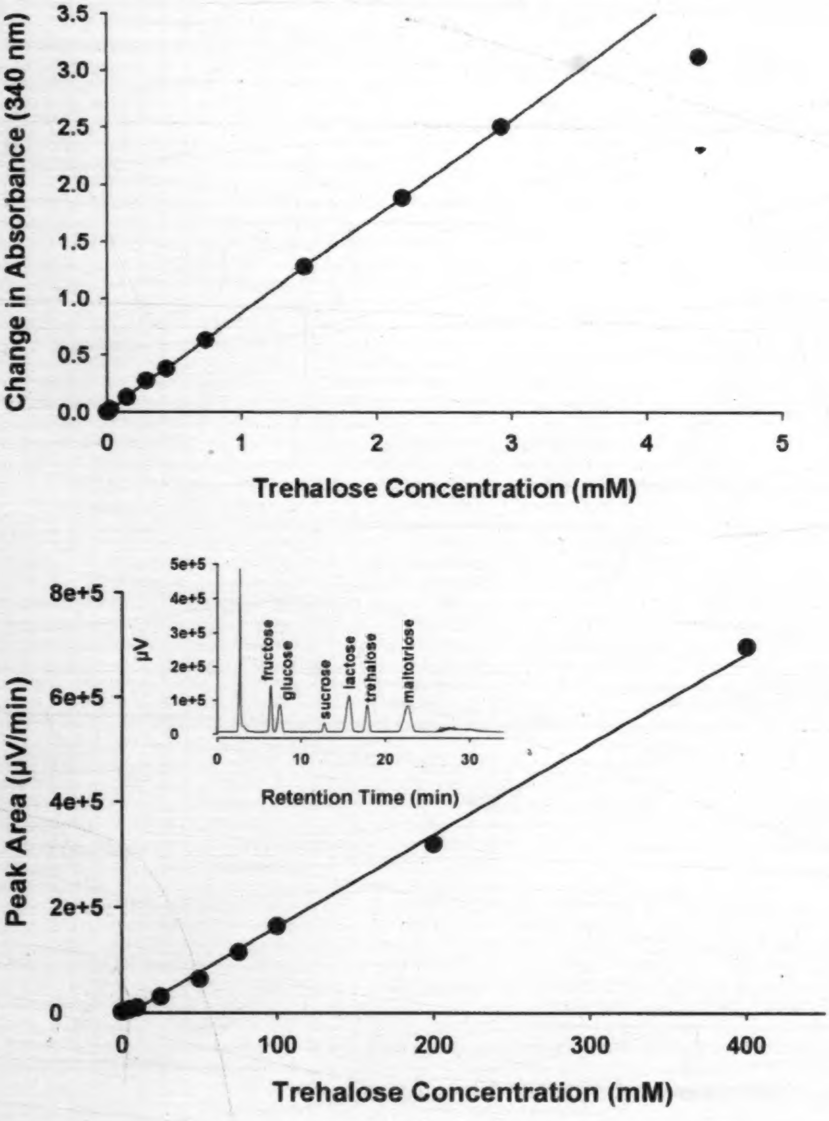


Figure 3.7. The spectrophotometrically-measured trehalose concentration of the 1 mM lipid liposomal lysates.

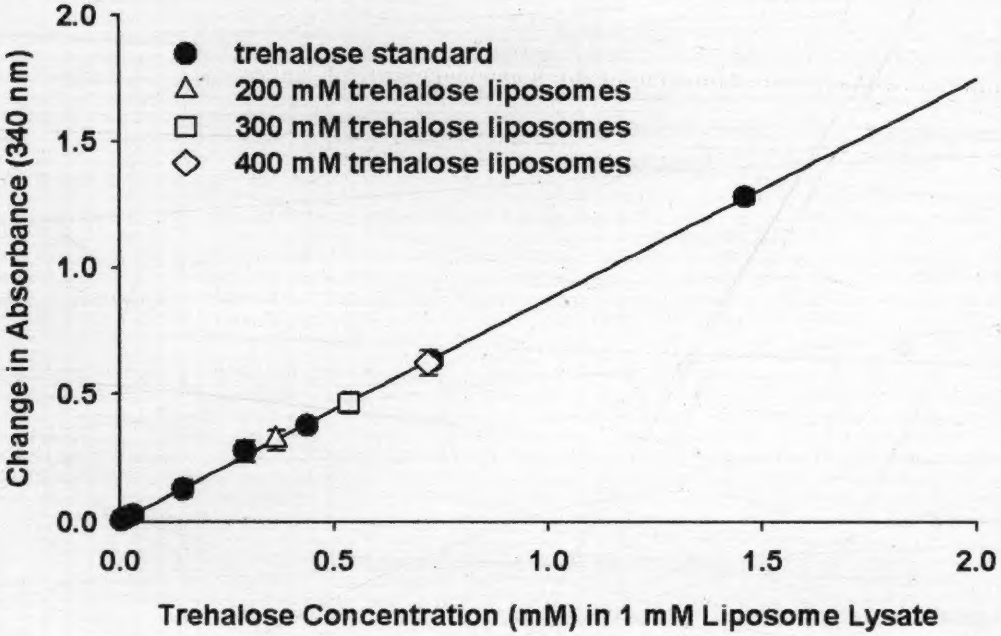


Figure 3.8. The spectrophotometrically-measured trehalose concentration of the 1 mM lipid liposomal lysates were confirmed with the HPLC measurements.

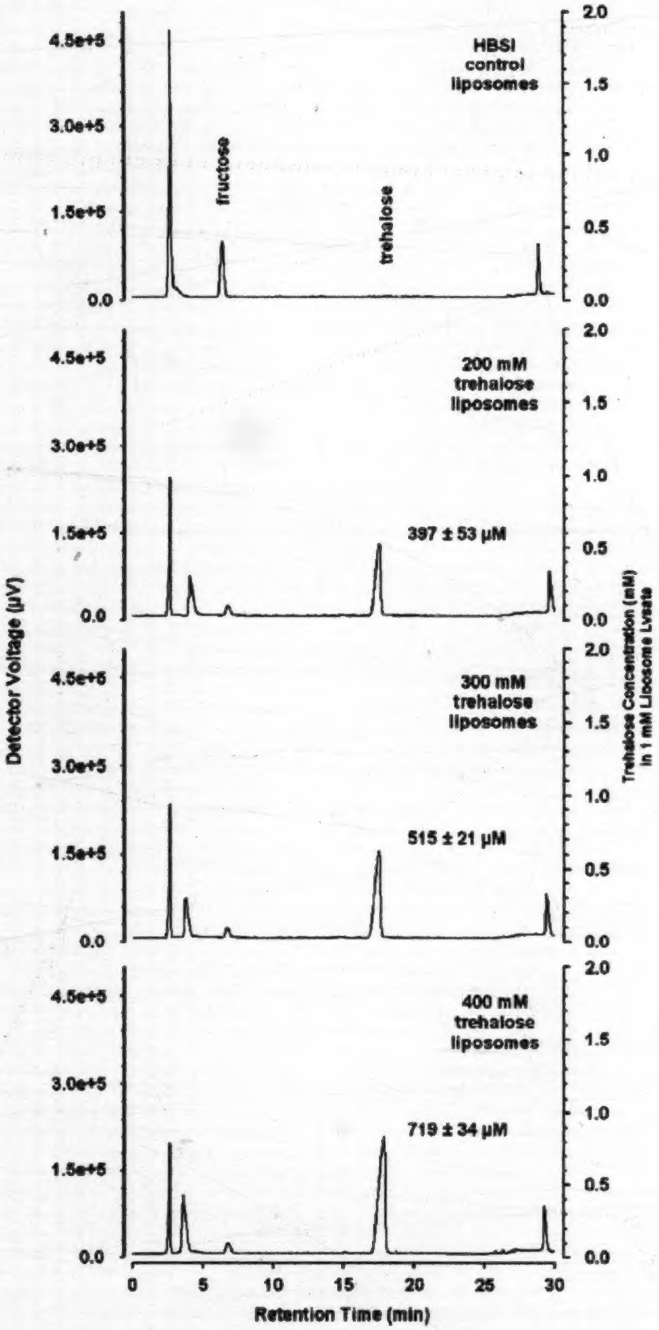
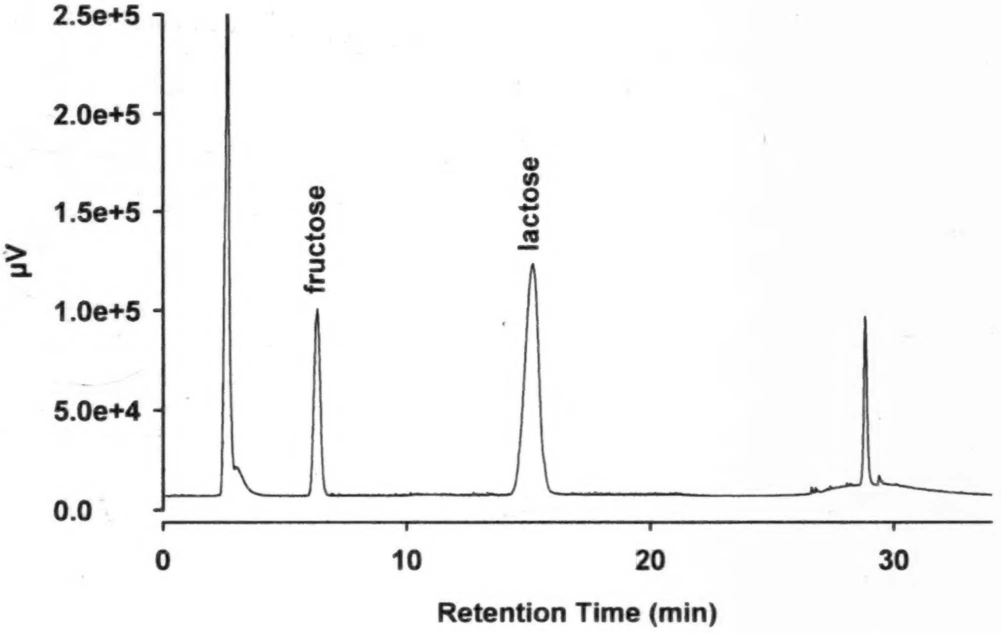


Figure 3.9. HPLC was performed on the liposomal supernatants prior to lysis in order to assess whether trehalose was only present in the liposomal aqueous core. Fructose and lactose were used as internal standards.



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A.1. Appendix for Section 3.2.2.4.

This appendix illustrates an example of calculations used to translate trehalose concentrations spectrophotometrically measured in 1 mM liposomal lysate of one preparation of liposomes hydrated by 200 mM trehalose buffer to intra-liposomal trehalose concentration and intra-liposomal encapsulation efficiency value.

moles of trehalose = (measured trehalose concentration in 1 mM lipid solution) x (solution volume)

ex. 163.6 mM x 2.2 mL = 0.360 mmol

$$\frac{\text{moles trehalose in 1mM lipid solution}}{\text{number of liposomes in 1mM lipid solution}} \times \frac{1}{\text{liposome core volume}} = \text{intra-liposomal trehalose concentration}$$

$$\text{ex. } \frac{0.360\text{mmol}}{6.32 \times 10^{13}\text{ liposomes / L}} \times \frac{1}{3.1 \times 10^{-17}\text{ L}} = 183.7\text{mM}$$

$$\frac{\text{intra-liposomal trehalose concentration}}{\text{hydrating buffer trehalose concentration}} \times 100\% = \text{trehalose encapsulation efficiency}$$

$$\text{ex. } \frac{183.7\text{mM}}{200\text{mM}} \times 100\% = 91.9\%$$

Chapter 4*

Investigating Interactions of Trehalose-containing Liposomes with Human Red Blood Cells

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4.1. Introduction

Following the initial report by Bangam *et al.*[1] that aqueous dispersions of phospholipids form relatively impermeable lipid vesicles dubbed liposomes, numerous articles have focused on examining liposome interactions with biological milieu *in vivo* and *in vitro*.[2; 3; 4; 5; 6; 7] The mechanism of such interaction has become central to many of the applications of liposomes, especially in the biomedical field. For example, in order to achieve therapeutic effectiveness of a drug-carrier liposome, it is essential to study liposome interaction with blood, the tissue and immune system, as wells as the specific interactions with a target cell. A majority of the liposome-cell interaction studies to date have been performed *in vitro* to avoid the additional complexities of an *in vivo* system, and have identified several modes of liposome-cell interaction: adsorption to cell membrane with or without lipid transfer, fusion with cells and endocytosis.[2; 3; 5; 6]

Adsorption is the association of intact liposomes with the cell membrane, without internalization. Liposomes can be loosely adsorbed to the cell membrane, in which case they can be removed by a simple wash, whereas tightly adsorbed liposomes require further processing to be released from the cell membrane.[2; 3; 8; 9] Liposome adsorption may be nonspecific, when mediated by such forces as electrostatic or hydrophobic, or specific, when mediated by a surface receptor or an antibody.[8; 9; 10] A variety of experimental techniques have been used to demonstrate liposome adsorption to cell membranes. For example, Pagano and Takeichi used SEM to show liposomes tightly adsorbed to the surface of Chinese hamster fibroblasts.[9] Radiographic and fluorescence studies confirmed the presence of vesicles at the cell surface, as well as that the adsorbed vesicles remain intact.[11; 12] Liposome adsorption is usually associated with the transfer of lipid molecules from the liposome membrane to the plasma membrane, without transfer of liposome aqueous contents.[8; 13] This type of interaction has also been described as "hemi-fusion".[8; 10; 14] Liposome lipids are not only transferred to the plasma membrane, but there is also a lipid exchange between the liposome and plasma membranes, as demonstrated by Pegano *et al.* using radiolabelled phospholipids, and Eytan *et al.* using fluorescently-labelled phospholipids. [5; 15] Martin *et al.* confirmed these findings by demonstrating incorporation of vesicle lipid into the plasma membrane of human red blood cells without content transfer.[13]

Similar to lipid transfer, fusion implies merging of the liposome and cell lipid bilayers. However, another criterion for the fusion is transfer of liposome aqueous core into cell cytoplasm. Therefore, membrane fusion requires at least three distinct processes: aggregation or binding of two membranes, the subsequent destabilization and merger of these membranes, and finally, mixing of the aqueous compartments.[16; 17] It has therefore been suggested that fusion occurs in combination with lipid transfer, secondary to the formation of the intermediate hour-glass shaped single bilayer structure.[2; 8; 16; 18; 19] The juxtaposition of lipid bilayers and the overall negative curvature of the single bilayer hemi-fusion structure leads to formation of a fusion pore that allows mixing of aqueous contents.[17; 20; 21] A variety of methods have been used to monitor liposome fusion to cells, including TEM to visualize fusion events;[19; 22] electron spin resonance and radiography to monitor the dilution of labelled liposome components into cells;[23; 24; 25] differential scanning calorimetry to investigate changes in phase transition temperatures of membranes due to lipid intermixing;[26] and more recently, fluorescence assays to monitor the intermixing of lipid membranes and/or the intermixing of aqueous contents of the fused entities.[8; 18; 27; 28; 29; 30]

Finally, the fourth mode of liposome-cell interaction is endocytosis, which can be defined as the uptake of intact liposomes into endocytic vesicles. Evidence for endocytosis was provided by Huang *et al.* whose TEM showed liposomes in lysosomes;[31] Magee *et al.*, who demonstrated recovery of the liposome components from subcellular fractions;[32] and Batzri *et al.*, who demonstrated that liposome uptake can be markedly reduced by applying endocytoic or metabolic inhibitors.[3]

It is important to note that, although the definitions of these mechanisms of liposome-cell interactions are straight-forward, the occurrence of these events is not. Multiple combinations of liposome-cell interactions may be occurring under a given experimental circumstance.[5; 6; 33] Therefore, it is difficult to unequivocally distinguish the liposome-cell interaction events, even with current advances in research analytical techniques.[8; 20; 27]

This thesis is investigating liposomal delivery as a permeabilization strategy for the intracellular accumulation of trehalose into human RBCs. The previous chapter of the thesis has focused on adapting liposome techniques to synthesize and characterize trehalose-containing liposomes for applications in biopreservation, so the next step, and the purpose of this chapter, is to examine interactions of the synthesized trehalose-containing liposomes with human RBCs. Delineating the mechanism of liposomal interaction with RBCs is an important step towards establishing the use of liposomes as tools for the intracellular delivery of trehalose. As mature RBCs are unable to endocytose,[34] there are three possible outcomes: no interaction, liposome adsorption to RBC membranes, and trehalose delivery through liposome fusion (Figure 4.1). We hypothesize that there is an interaction between trehalose-containing liposomes and RBCs, and that the mechanisms of interaction includes both adsorption of the vesicles to the RBC surface and liposomal incorporation through membrane fusion.

4.2. Materials and Methods

4.2.1. Synthesis and Characterization of Fluorescently-labelled Trehalose-containing Liposomes

Three types of unilamellar fluorescently-labelled liposomes were synthesized using an extrusion procedure described in Chapter 3: R18 liposomes, 5(6)-CF liposomes, and 5(6)-CF-R18 liposomes. In addition, one batch of unmarked 200 mM trehalose liposomes was synthesized as a control.

R18 fluorescent marker was added to DPPC:cholesterol mixture (66:30 mol %) by injection during vortexing in the first step of liposome synthesis, to self-quenching final concentration of 4 mol % with respect to total lipid, followed by a

15 min incubation at room temperature in the dark.[35] Lyophilized R18-labelled lipids were then hydrated with 200 mM trehalose buffer (please refer to Table 3.1 for exact compostion, pH and osmolality), followed by previously described steps for synthesis of unilamellar liposomomes (Chapter 3). In contrast to using R18 to label the liposome membrane, 5(6)-CF lipophobic fluorophore was added to label the liposome aqueous core (Molecular Probes, Invitrogen, ON, Canada). 5(6)-CF liposomes contained phospholipid and cholesterol lipid bilayer (70:30 mol %) were hydrated with 200 mM trehalose buffer (Table 3.1), containing 20 µM 5(6)-CF (Molecular Probes, Invitrogen, ON, Canada). Finally, the third type of fluorescent liposomes, 5(6)-CF-R18 liposomes, were synthesized to contain both R18 marker incorporated in lipid bilayers and 5(6)-CF marker in the aqueous core.

Unincorporated fluorescence markers were removed from liposome solution by passing the liposome solution through a Sephadex G-75 (1 x 15 cm) column (Amersham Biosciences, Uppsala, Sweden) with 140 mM NaCl, 20 mM HEPES washing buffer as eluent. Free marker strongly adsorbs to the top of the column, whereas labelled liposomes can be recovered in the void volume fraction.[36] After washing and resuspension procedures, synthesized fluorescently-labelled, trehalose-containing liposomes and control liposomes were characterized for lipid content, size and trehalose content, according to assays described in Chapter 3. In addition, liposome population distribution, homogeneity and relative fluorescence were examined using FACSCalibur flow cytometer (BD Sciences, San Jose, CA) equipped with an argon laser and computer software (CellQuest BD Sciences, San Jose, CA).* Initial gating of the liposome population was set using 400 nm standard latex beads (Bangs Laboratories Inc., Fishers, IN) and the analysis of the forward scatter (FSC) and the side scatter (SSC) data on the flow cytometer. R18 fluorescence intensity was measured in the FL-2 channel (excitation 550 nm, emission 590 nm), while 5(6)-CF fluorescence intensity was measured in the FL-1 channel (excitation 445 nm, emission 525 nm). For each liposome sample, 20 000 events were collected at a constant flow rate.

4.2.2. Liposome Incubation with RBCs

Human blood (7 mL) was collected from healthy volunteers into vacutainer tubes containing citrate anticoagulant (BD, Fanklin Lakes, NJ) using standard phlebotomy, according to CBS protocols. The sample was centrifuged at 1500 x g for 10 min at 4 °C (Eppendorf Centrifuge 5810R, Westbury, NY). Plasma supernatant and buffy coat were removed and the RBC pellet was washed three times with HBS physiological buffer (Table 3.1). After the last wash, RBCs were resuspended in 5 mL of HBS buffer, counted on a hematology cell analyzer (Beckman Coulter AcT, New York, NY), and diluted to an appropriate count for future experiments. Phlebotomized blood samples were stored refrigerated (4 - 6 °C) for up to 3 days until needed. The following incubation conditions were used for data presented in this chapter: fluorescently labelled liposomes (1 mM lipid) were incubated with RBC solution containing $1x10^7$ RBC/mL for 2 h at room temperature on a laboratory shaker with low speed setting (VWR International, West Chester, PA).[37] Other incubation conditions were also examined and the effects of changing experimental parameters during incubation on liposome-RBC interactions are described in Chapter 5.

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* The author would like to gratefully acknowledge Dorothy Drutkowski's assistance with flow cytometry at the Flow Cytometry Facility, University of Alberta.

4.2.3. Density Gradient Separation

After incubation, density gradient separation was used to separate RBCs from unbound liposomes that were adsorbed to the RBC membranes, along with the free liposomes in the incubation mixture. Ficoll-Paque (GE Healthcare Biosciences, Bjorkgatan, Sweden) is a commercially available polyvinyl-coated colloidal silica solution commonly used for fractionation of biological samples based on particle size. RBC-liposome incubation mixture was carefully layered on an equal volume of Ficoll-Paque solution in a 15 mL centrifuge test tube and centrifuged at 300 x g for 30 min at room temperature, with low acceleration and brake speed settings. After centrifugation, the supernatant containing the liposomes was removed and the RBCs pellet was washed with HBS buffer and resuspended in the appropriate experimental solution.

4.2.4. Monitoring Liposome Interactions with RBCs

Flow cytometry, fluorescent microscopy and spectrophotometry were used to assess the interactions between fluorescently labelled liposomes and RBCs. Fluorescently-labelled liposomes were removed from RBCs after incubation using density gradient separation before examining the cells with a fluorescent microscope, performing spectrophotometric trehalose measurement, and in some instances, before flow cytometry.

Flow cytometry uses the principles of light scattering, light excitation and emission to generate multi-parameter data from particles suspended in a sheath of phosphate buffered saline fluid. The flow cytometry program was set to acquire 20,000 events at a medium flow rate. A region around the RBC population was set using fresh RBCs before incubation with liposomes. The results were expressed in arbitrary units of R18 and 5(6)-CF fluorescence, in a single-parameter histogram, where the horizontal axis represents the fluorescence intensity, and vertical axis represents the number of events per channel.

After incubation with 5(6)-CF and R18 liposomes and density gradient separation, RBC samples were fixed with 1 % formaldelyde (Catlag Laboratories, Burlingame, CA) and coverslipped. RBCs then were examined on a Eclipse 80i fluorescent microscope (Nikon Inc. Melville, NY) with Orca-ER digital CCD camera (Hamamatsu Photonics, Bridgewater, NJ) and Wasabi ver4.1 software (Hamamatsu Photonics, Bridgewater, NJ) in Acker Research Lab. Four-laser confocal microscope (LSM510 Carl Zeiss MicroImaging Inc., Thornwood, USA) with MetaMorph ver7.5 software (Molecular Devices, Sunnyvale, CA) at the Cross Cancer Cell Imaging Facility was used to examine RBCs after interacting with 5(6)-CF-R18 liposomes (green fluorescence filter excitation 440 nm, emission 520 nm, red fluorescence filter excitation 560 nm, emission 590 nm).[*]

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[*] The author would like to gratefully acknowledge Jennifer Dien Bard's assistance with confocal microscopy at the Cross Cancer Cell Imaging Facility.

In addition to examining transfer of fluorescence from liposome to RBC populations, the delivery of liposomal contents from R18-liposomes, 5(6)-CF liposomes, and control 200 mM trehalose liposomes into RBCs was assessed by spectrophotometric measurement of intracellular trehalose concentrations. After density gradient separation and washing, RBCs were resuspended in HBS buffer and run on a hematology analyzer (Coulter AcT, Beckman Coulter, New York, NY) to obtain a count and mean cell volume. RBCs were then lysed by immersing the solution into liquid nitrogen and slow thawing at room temperature three times, which has shown to result in hemolysis near 100 %. Trehalose concentration in solution was measured using Megazyme method, as described in Chapter 3. Intracellular trehalose concentration was calculated based on measured trehalose in solution, RBC count, and RBC volume corrected for the osmotically inactive fraction, as described in Appendix A.2. RBC osmotically inactive fraction value of 0.423 used in these calculations was obtained by averaging three values reported in the literature.[38; 39; 40]

4.3. Results

Physical characteristics of fluorescently-labelled liposomes and control liposomes are summarized in Table 4.1. While 5(6)-CF liposomes showed similar characteristics to control liposomes, liposome preparations containing R18 marker had lower mean lipid concentration. R18-containing liposomes required an additional passage through a Sephadex G-75 column and two additional washes compared to 5(6)-CF-liposomes to ensure adequate removal of unincorporated marker, which may explain lower lipid recovery of these liposomes. The size analysis revealed a unimodal Gaussian population distribution of liposomal preparations (similar to trehalose-containing liposomes illustrated in Figure 3.4). While the size distribution of R18-containing liposomes was not significantly different from the other liposomes (p=0.515, nonparametric Kruskal-Wallis ANOVA), the larger standard deviation values may reflect uneven distribution of R18 marker in the liposome lipid bilayers. The size distribution of R18-containing liposomes was somewhat wider, which might reflect uneven distribution of R18 marker in the liposome lipid bilayers. The average sizes of the polystyrene and silica standard sizing beads used as controls were 147 ± 39 nm for the 200 nm beads, 324 ± 52 nm for the 400 nm beads and 766 ± 78 nm for the 800 nm beads. Mean intra-liposome trehalose concentrations of fluorescently-labelled liposomes suggest that fluorescent markers did not interfere with encapsulation of trehalose inside the liposome core.

Results of R18 liposomes interacting with RBCs are shown in Figure 4.2. R18 fluorophore was introduced to liposome lipid bilayer at high concentration, resulting in low fluorescence emission of liposome population, as shown by the fluorescence histogram. However, after RBCs were incubated with R18 liposomes, the RBC population showed high florescence emission, with mean fluorescence intensity 344 ± 11 % higher than initial R18 liposome mean fluorescence intensity (Figure 4.2). These results suggest dequenching of R18 marker in RBC membrane upon liposome association with RBCs. The delivery of R18 marker appears to be homogeneous, as 99 % of RBC population became fluorescent. Density gradient separation resulted in significant removal of R18 liposomes, evident by a 94 % decrease in number of events in liposome gate (Figure 4.3). Density gradient separation also resulted in 32 % decrease in mean fluorescence intensity of R18-labelled RBCs, suggesting that initial transfer of liposome fluorescence is largely due to R18 liposomes loosely adsorbing to RBC membrane (Figure 4.3). Fluorescent microscopy image of RBCs after interaction R18 liposomes demonstrates homogeneous incorporation of R18 marker into the RBC membrane (Figure 4.4).

Flow cytometry results of 5(6)-CF liposomes and RBCs before the incubation step are shown in Figure 4.5, and after the incubation step in Figure 4.6. Before incubation, RBC population showed typical scatter profile with no fluorescence, while flow cytometry data confirmed liposome population with entrapped 5(6)-CF fluorescent marker. After the incubation step, there is an appearance of fluorescent RBC population, paralleled by the shift of RBC fluorescence histogram to the right, which suggest the association of 5(6)-CF liposomes with human RBCs. Similar to R18 liposomes, density gradient separation resulted in marked removal of liposomal population, evident by a 97 % decrease in number of events in liposome gate (Figure 4.7). However, in contrast to RBCs interacting with R18 liposomes, density gradient separation had a large effect on mean RBC fluorescence intensity, which decreased 81 %, suggesting that initial RBC fluorescence is largely due to 5(6)-CF liposomes coating RBC membrane. Figure 4.8 shows fluorescent microscopy image of

RBCs after interacting with 5(6)-CF liposomes. RBCs appear fluorescent, with relatively uniform distribution of 5(6)-CF marker.

Changes in fluorescence intensity of RBC population after interacting with 5(6)-CF-R18 liposomes can be seen in Figure 4.9. Flow cytometry and confocal microscopy images show transfer of both hydrophilic and hydrophobic fluorescent markers from liposome population. While green 5(6)-CF marker of liposome core appeared localized to RBC cytoplasm (Figure 4.9.B), red R18 marker appears to have incorporated itself into RBC membrane (Figure 4.9.C). Orange color around RBC membrane indicates presence of both fluorescent markers in the area of RBC membrane (Figures 4.9.C and 4.9.D).

Liposomal content delivery demonstrated by transfer of 5(6)-CF marker was confirmed by spectrophotometric measurement of intracellular trehalose, as shown in Table 4.2. Liposomes permeabilized RBC plasma membrane to deliver micromolar concentrations of trehalose to RBC cytosol. RBCs incubated with R18 liposomes showed statistically significant higher trehalose concentrations compared to RBCs incubated with 5(6)-CF or unmarked liposomes ($p=0.027$, nonparametric Kruskal-Wallis ANOVA).

4.4. Discussion

In the early 1970s, Papahadjopoulos *et al.* provided convincing evidence that liposomes fuse with cell membranes by demonstrating cellular uptake of two liposome markers in identical amounts, liposome membrane radiolabel and a different radiolabel trapped inside liposomes, after inhibiting cellular endocytotic pathways.[23; 24] Liposome fusion with RBC membranes has been also elegantly demonstrated by Martin *et al.*, using a series of freeze-fracture TEM images of vesicle–plasma membrane fusion event.[19] The emergence of a broad repertoire of fluorescent probes has lead to the introduction of new assays, such as fluorescence dequenching and resonance energy transfer, which has enabled further examination of the details of the fusion process.[14; 41; 42; 43] Due to their sensitivity, reliability, cost and convenience, fluorescence assays are currently the most widely used methods in liposome fusion studies.[8; 14; 41; 43]

In this study, a lipid mixing assay based on fluorescence dequenching of R18 was used to monitor the merging of liposome-RBC membranes, while 5(6)-CF marker was used to examine the intermixing of aqueous contents. R18 dequenching assay is the most widely used method to monitor membrane fusion.[44] This marker has been shown to easily redistribute into lipid membranes at high self-quenching concentrations, with efficiency of probe incorporation on the order of 70 %.[42; 45; 46] Also, R18 is much less likely to interfere with the fusion process, compared to the bulky fluorescently-labelled phospholipids.[14; 43; 47] Upon merging with unlabelled target membranes, dilution of the R18 probe takes place with a concomitant increase in fluorescence that allows the measurement of lipid mixing, which is an essential event in the fusion process. The results of the R18 dequenching assay demonstrated transfer of R18 marker from the membranes of trehalose-containing liposomes, shown by a marked increase of RBC fluorescence compared to liposomes. These results, combined with convincing fluorescence microscopy images, provided evidence that trehalose-containing liposomes are interacting with human RBCs. In addition, these results are strongly indicative of lateral diffusion of liposome membrane phospholipids into the RBC lipid bilayer. Therefore, trehalose containing liposomes are probably adsorbing to the RBC membrane, which involves transfer of lipid molecules from liposome membrane to the plasma membrane. Density gradient separation results suggest that liposomes are adsorbing both loosely and tightly to RBC surface. An alternative explanation that the significant increase in RBC fluorescence might simply represent multiple R18 liposomes interacting with one RBC is not consistent with the flow cytometry results of RBCs interacting with 5(6)-CF liposomes, where the mean fluorescence intensity of 5(6)-CF liposomes is consistently higher than the fluorescence intensity of RBCs after the interaction, although RBCs are expected to interact with many liposomes. Multiple-fold increase in fluorescence intensity is a hallmark of dequenching.[45; 46; 48]

Although R18 dequenching suggests transfer and redistribution of liposome phospholipids into RBC plasma membrane, we cannot be certain that trehalose-containing liposomes are actually fusing with RBCs. It is possible that the dequenching of R18 marker resulted from molecular exchange of R18 between membranes, with or without formation of the intermediate single bilayer structures in which the outer monolayers of vesicles and cell plasma membranes are transiently merged. Probe transfer mechanisms due to aggregation, collision, passive probe diffusion or the action of lipid-transfer proteins have been previously described.[46; 49; 50] Therefore, it is important to distinguish these events from a true fusion interaction, which implies delivery of liposomal aqueous contents.

To answer the question whether trehalose-containing liposomes are actually fusing with RBCs, an aqueous content mixing assay was performed by labelling the liposome aqueous core with a fluorescent marker, 5(6)-CF, and observing the fluorescence transfer. 5(6)-CF was chosen to label the intra-liposomal aqueous core because it has many similar characteristics to trehalose: molecular weight (376 g/mol vs. 378 g/mol, respectively), neutral charge, high solubility in aqueous solutions and impermeability to plasma membranes. Therefore, we expect similar intra-liposome encapsulation efficiency and delivery of this fluorescence marker to trehalose (Chapter 3). The flow cytometry and fluorescence microscopy results of the aqueous content mixing studies strongly suggest, but do not prove, that trehalose containing liposomes are indeed fusing with human RBCs. This finding is supported by the fluorescence transfer from 5(6)-CF-R18 liposomes to RBCs and detection of intracellular trehalose concentration. Therefore, in addition to adsorption and lipid transfer, trehalose-containing liposomes are likely fusing with human RBCs. It is unknown whether adsorption and/or fusion of trehalose-containing liposomes is only mediated by nonspecific forces, as recent literature suggests presence of specialized membrane proteins in multiple cell types promoting membrane aggregation and fusion.[21; 29; 30; 33] The presence of such proteins in human RBCs has not yet been demonstrated. Although liposome fusion is a more likely explanation for the obtained results, it is possible that upon liposome adsorption, both liposome and RBC membranes become destabilized, which may be followed by extracellular release of the liposome contents and subsequent passive transport of 5(6)-CF and trehalose into RBCs.

The difference in the flow cytometry results before and after density gradient separation suggest that liposome coating of RBC membranes is contributing considerably to RBC fluorescence intensity. There was a marked difference between the decrease in RBC fluorescence intensity after density gradient separation of RBCs interacting with 5(6)-CF liposomes and R18 liposomes, although this process appears to have removed most of both types of fluorescently labelled liposomes (Figures 4.3 and 4.7). In addition, 5(6)-CF liposomes delivered similar amounts of trehalose to RBCs compared to unmarked liposomes, while measured trehalose was much higher in RBCs interacting with R18 liposomes (Table 4.2). The properties of R18 marker might explain these findings. It has been previously reported that a proportion of the R18 marker can be associated with membranes in the form of micelles and clusters, rather than randomly distributed as monomers in the membrane.[49; 51] Size evaluation of R18 trehalose-containing liposomes did show smaller mean particle size and wider size distribution of these vesicles compared to unmarked and 5(6)-CF liposomes (Table 4.1). R18 micelles and clusters may lead to irregularities in the liposomes lipid bilayer, and it has been previously shown that increased membrane irregularity promotes membrane aggregation and formation of hemi-fusion intermediate structures.[21; 33; 42] Therefore, R18 liposomes might be more tightly adsorbed to the RBC membrane compared to 5(6)-CF and unmarked liposomes, and therefore more resistant to removal by density gradient separation, explaining why fluorescence intensity of RBCs incubated with R18 liposomes does not markedly decrease after density gradient separation. Hence, in this case, trehalose measurement is probably reflecting not only RBC intracellular concentration, but also trehalose encapsulated within the R18 liposome aqueous core.

Membranes do not hemi-fuse spontaneously; the repulsive energy between two opposing phospholipid membranes in an aqueous environment needs to be overcome. The exact mechanism of this event is still one of the major questions in biology.[21] An attractive force is believed to arise when hydrophobic phospholipid areas are exposed due to the stress exerted on the bilayer surfaces, allowing the lipid bilayers to approach one another within nanometer distances.[20; 33] In addition, a theoretical analysis of the interactions between apposed membranes at short separations suggests that thermal fluctuations of the lipid bilayers lead to the formation of a close contact, and the increasing hydration repulsion between the apposed phospholipid polar heads causes the rupture of interacting monolayers. This leads to the formation of hemi-fusion "single bilayer septum" intermediate in which the outer monolayers of vesicles and cell plasma membranes are transiently merged.[2; 8; 18; 21] The stability of these hemifusion intermediates is still debatable.[20; 21; 33]

Both fluorescence and spectrophotometry results suggest that liposomes can be used to permeabilize RBC membranes and to deliver trehalose to the RBC cytosol. However, liposomes delivered only micromolar concentrations of trehalose. As mentioned in the introduction section, there is no consensus on the intracellular trehalose concentration needed to confer biostabilization to mammalian cells. However, most of the literature shows that beneficial results are seen after intracellular loading of milimolar trehalose concentrations.[52; 53; 54] de Castro and Tunnacliffe have reported that 80 mM intracellular trehalose was insufficient to protect mouse fibroblasts during drying.[54] Work with fibroblasts has shown that an internal trehalose concentration between 100 – 200 mM is required to protect the membrane.[53] In the work by Crowe *et al.* on the freeze-drying of human platelets they report that their method of loading resulted in the intracellular accumulation of 17 to 18 mM when the platelets were incubated at 37 °C in 52 mM extracellular trehalose for 4 hours. As the concentration of intracellular protectant required will be dependent on the preservation conditions,[52] optimizing the delivery of intracellular trehalose will need to occur concurrently with more detailed studies on the low temperature response of the RBC.

Further calculations comparing number of moles of trehalose inside liposomes and RBCs after taking into account differences between liposome and RBC volume, indicate that the measured RBC intracellular trehalose concentration reflects fusion of less than 10 liposomes. There are multiple reasons for the low liposome fusion efficiency. For example, Crowe and colleagues have suggested that trehalose interacts with membrane phospholipids to maintain bilayer fluidity, presumably by hydrogen bonding, thus providing increased membrane stability leading to minimized membrane aggregation and fusion inhibition.[55; 56; 57] In addition, the occurrence of any of the liposome-cell interactions largely depends on liposomal physical characteristics, including composition, charge and size.[8; 58; 59] Other major determinants for the mechanism of liposome-cell interaction are cell type, and environmental factors, such as incubation conditions *in vitro*, or the presence of blood or serum *in vivo*.[8; 12; 37; 58; 59] The effects of experimental conditions on *in vitro* interaction of trehalose-containing liposomes with human RBCs and liposomal trehalose delivery efficiency will be further explored in the next chapter.

4.5. Conclusion

Delineating the mechanism of liposomal interaction with RBCs is an important step towards establishing the plausibility of using liposomes as tools for the intracellular delivery of stabilizing disaccharides for biopreservation purposes. The results from this thesis chapter including fluorescence transfer and intracellular trehalose measurement strongly support the hypothesis that liposomes can be used as trehalose delivery vesicles. The presence of liposome-RBC membrane lipid mixing and content delivery indicates that the mechanism of interaction between unilamellar trehalose-containing liposomes and human RBCs involves adsorption of the vesicles to the RBC surface, as well as liposome content incorporation through membrane fusion. Studies in the next chapter will address the experimental parameters affecting liposome-RBC interactions, trehalose delivery and RBC membrane quality.

Table 4.1. Physical characteristics of fluorescently-labelled and control liposomes synthesized in the study.

liposome preparation	mean liposome lipid concentration (mM)	mean liposome size (nm)	mean intra-liposome trehalose concentration (mM)
5(6)-CF liposomes	23.4 ± 1.6	403 ± 75	179.8 ± 11.1
R18 liposomes	18.9 ± 1.9	379 ± 101	177.3 ± 10.8
5(6)-CF-R18 liposomes	18.4 ± 2.1	388 ± 93	NA
control trehalose liposomes	24.0 ± 1.2	404 ± 82	182.7 ± 9.1

Table 4.2. Concentration of trehalose in red blood cells following interaction with liposomes. Delivery of intraliposomal content was confirmed by intracellular trehalose measurements (n=3).

liposome preparation	measured trehalose concentration (mM)	RBC count (x 10^{11}/L)	RBC mean corpuscular volume (fL)	calculated intracellular trehalose concentration (μM)*
5(6)-CF liposomes	2.57 ± 0.2	5.93 ± 0.1	91 ± 2	182 ± 31
R18 liposomes	13.4 ± 1.6	5.84 ± 0.2	92 ± 3	961 ± 74
control trehalose liposomes	3.75 ± 0.4	6.99 ± 0.1	91 ± 3	224 ± 19

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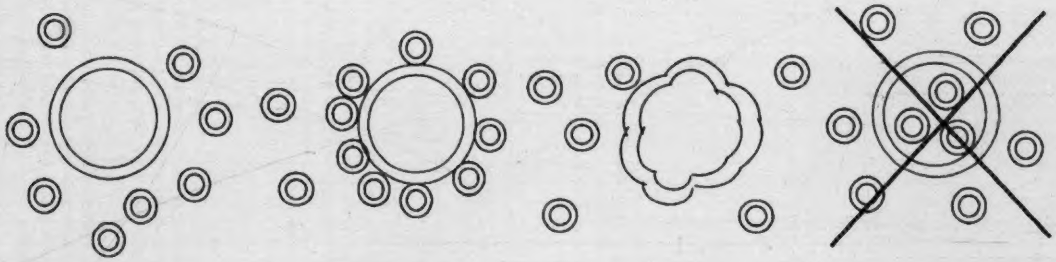
* Please refer to Appendix A.2. for example of calculations used to determine intracellular trehalose concentration.

Figure 4.1. Schematic of the possible modes of interactions between trehalose-containing liposomes and mammalian cells. As mature human RBCs are unable to endocytose, there are three possible outcomes: no interaction, liposome adsorbtion to RBC membranes, and trehalose delivery through liposome fusion.



no interaction | adsorption | fusion | ~~endocytosis~~

Figure 4.2. R18 trehalose-containing liposome interacting with RBCs. Red fluorescence (FL2-H) as a function of forward side scatter (FSC-H) (Figure A) depicts a highly fluorescent RBC population when mixed with the quenched R18 liposomes. The fluorescence histogram (Figure B) illustrates dequenching of R18 marker in RBC membrane upon transfer from liposome population.

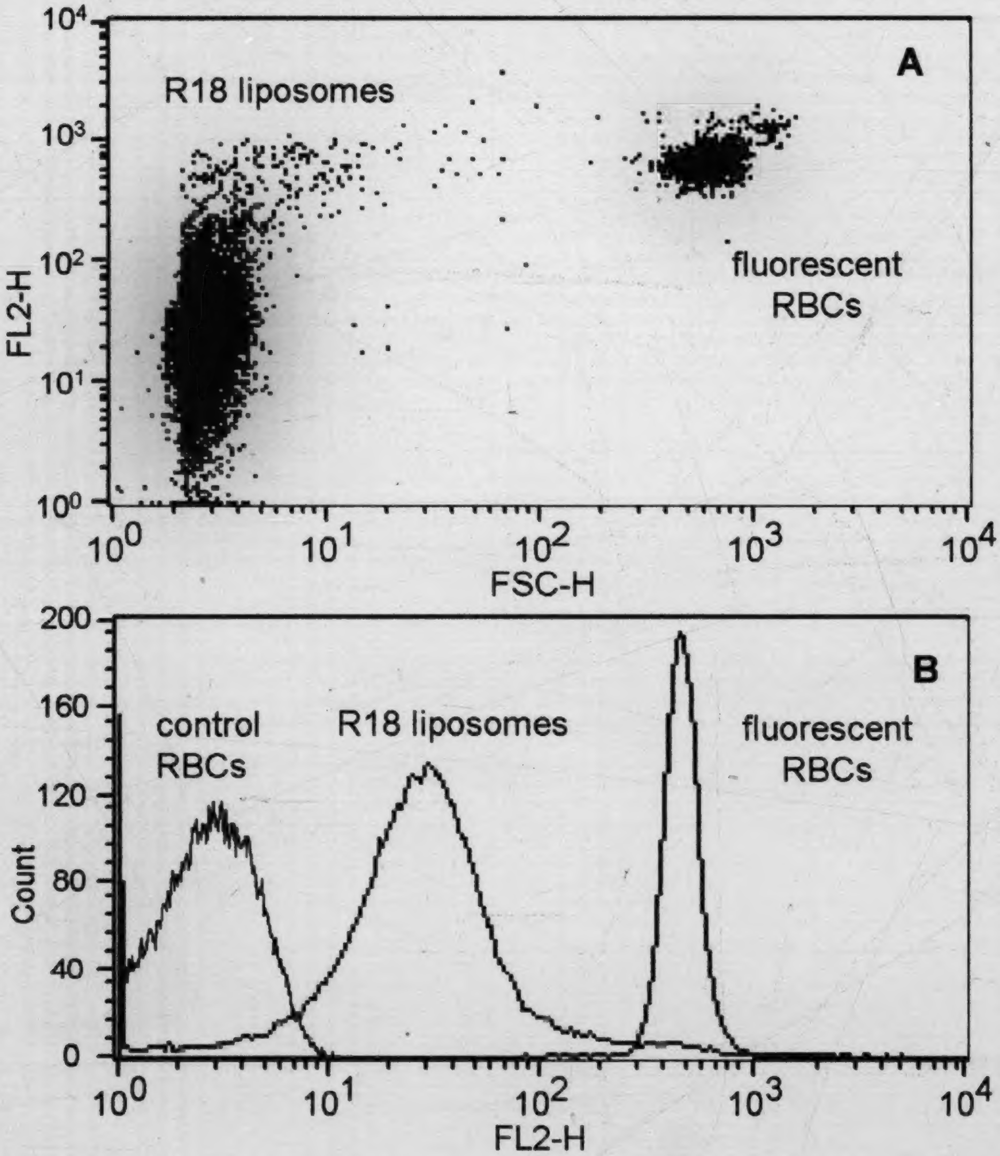


Figure 4.3. Effect of density gradient separation on R18 liposome interaction with RBC. Density gradient separation removed the majority of R18 liposomes (Figure B) but did not result in marked reduction of RBC mean fluorescence intensity (Figure A).

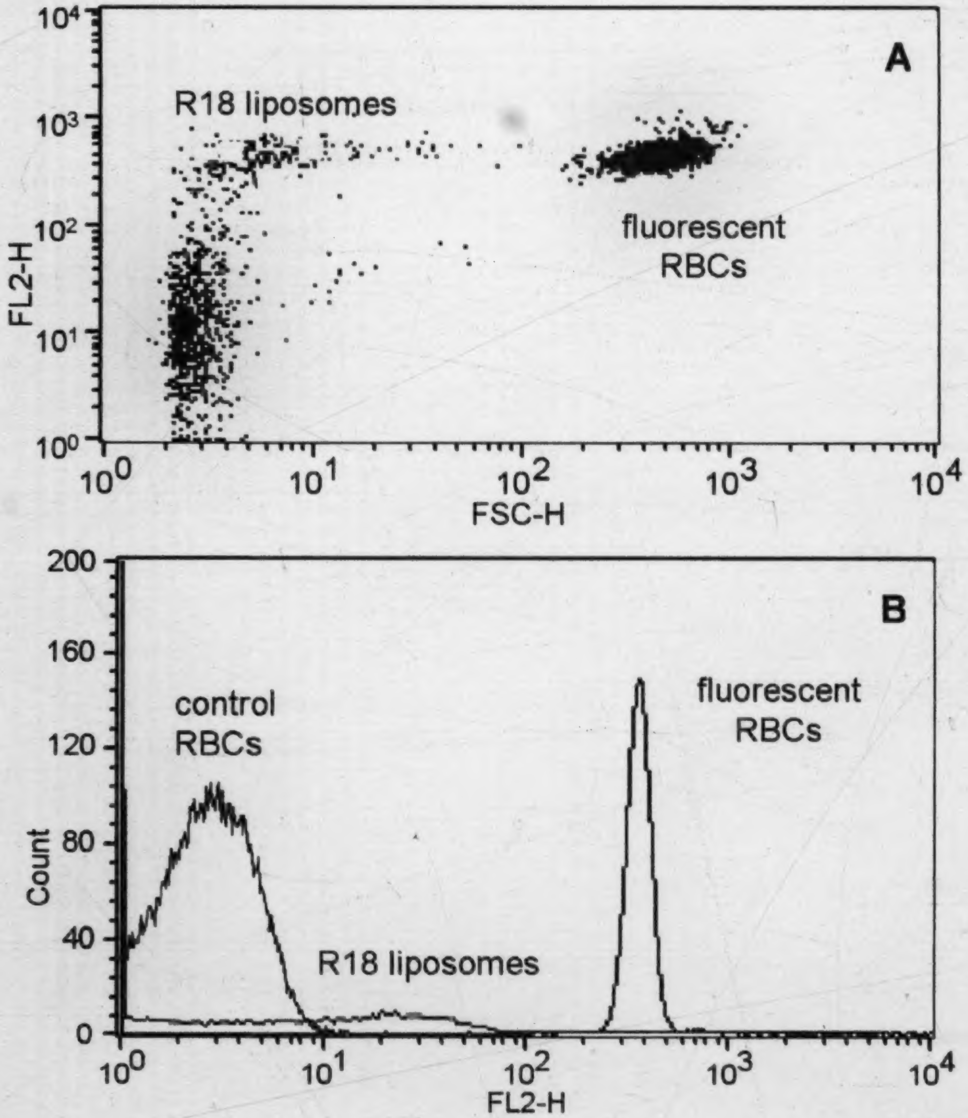


Figure 4.4. Fluorescent microscopy image of dequenching of R18 marker in RBC membrane after interaction with R18 liposomes.

Figure 4.5. Flow cytometric analysis of 5(6)-CF liposomes (Figure A) and control RBCs (Figure B) with corresponding fluorescence histograms (Figure C) before incubation and interaction. Green fluorescence (FL1-H) is plotted as a function of forward side scatter (FSC-H) (Figure A and B).

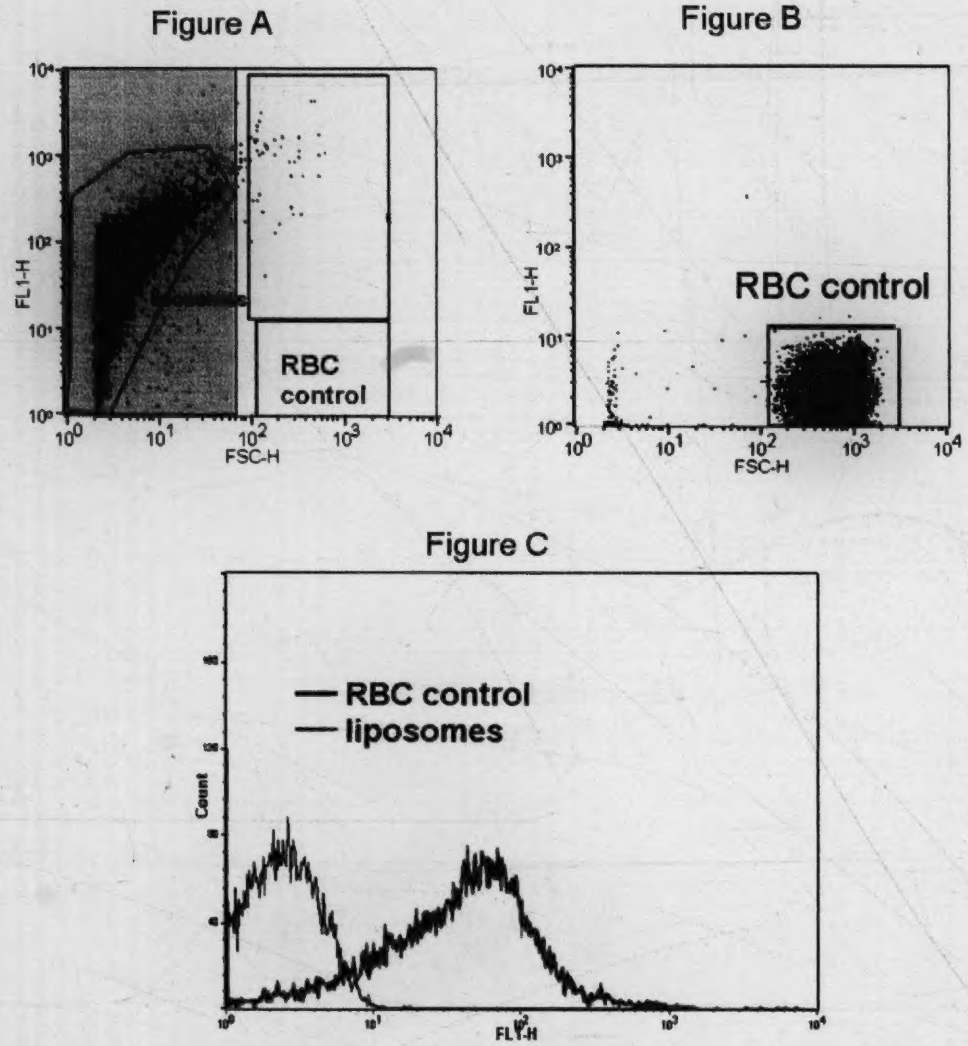


Figure A

Figure B

Figure C

Figure 4.6. Flow cytometric analysis of 5(6)-CF liposomes and RBCs after incubation (Figure A and B). The fluorescence histogram (Figure B) illustrates transfer of the 5(6)-CF to the RBC membrane resulting in fluorescent RBC.

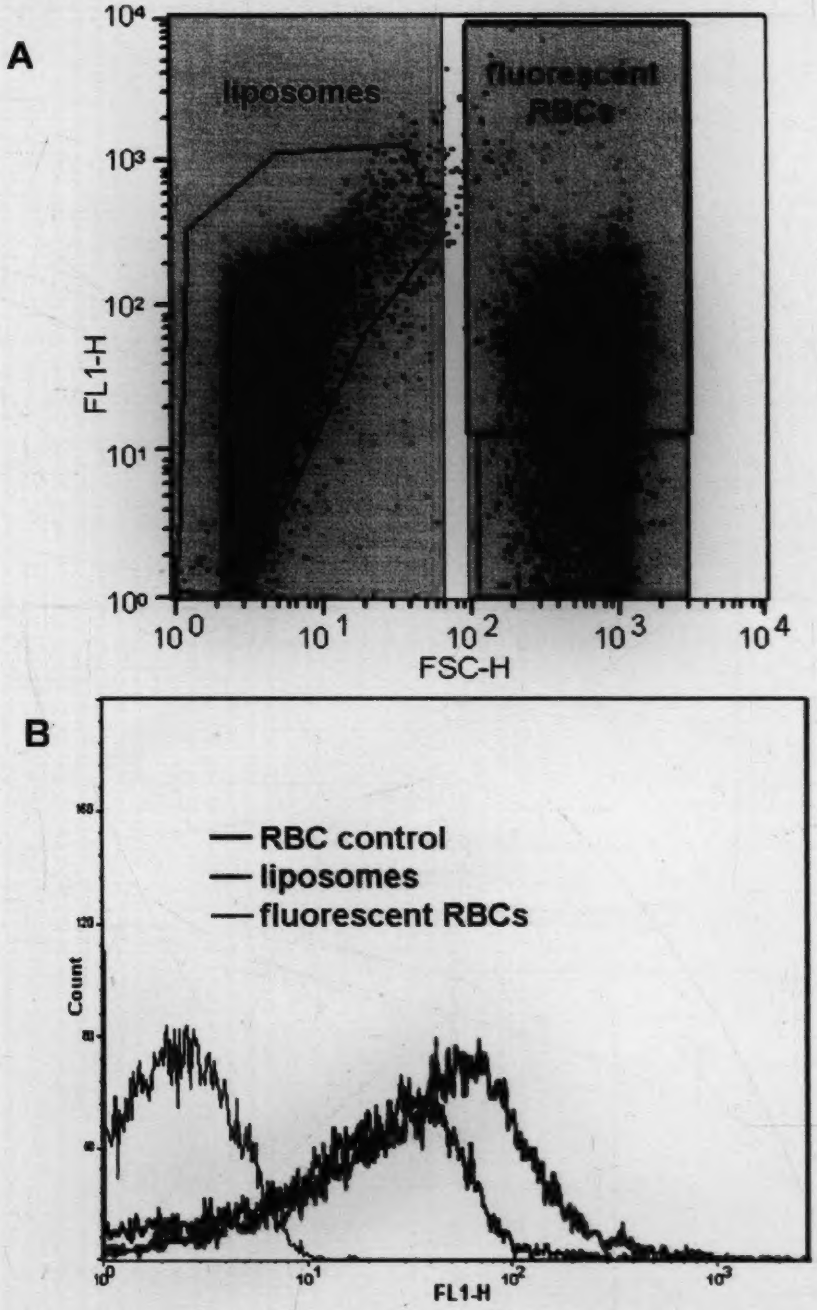


Figure 4.7. Effect of density gradient separation (Percoll) on the interaction of trehalose-containing liposomes with human RBC. Density gradient separation was effective at removing the majority of the 5(6)-CF liposomes from solution (Figure A), however it also resulted in a decrease in the transfer of 5(6)-CF to RBC (Figure B).

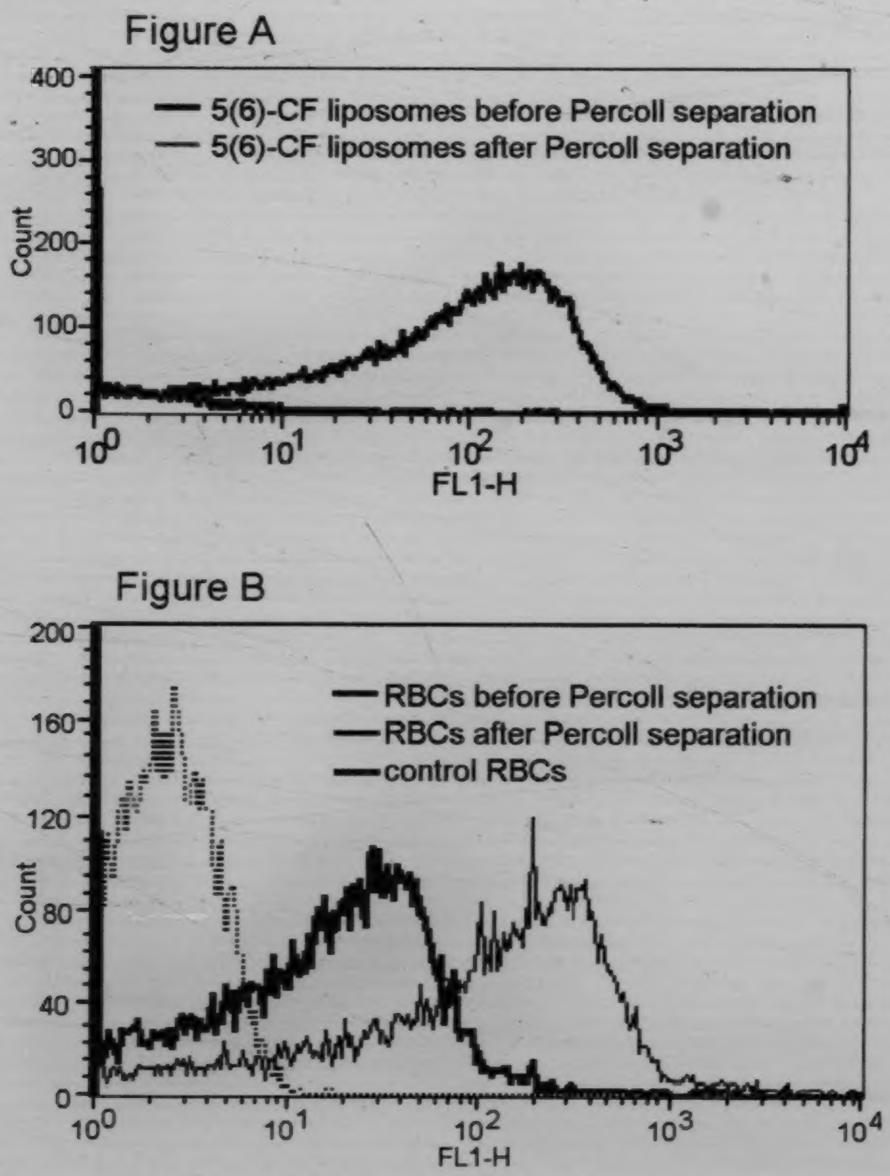


Figure 4.8. Fluorescent microscopy image of RBCs after interacting with 5(6)-CF liposomes.

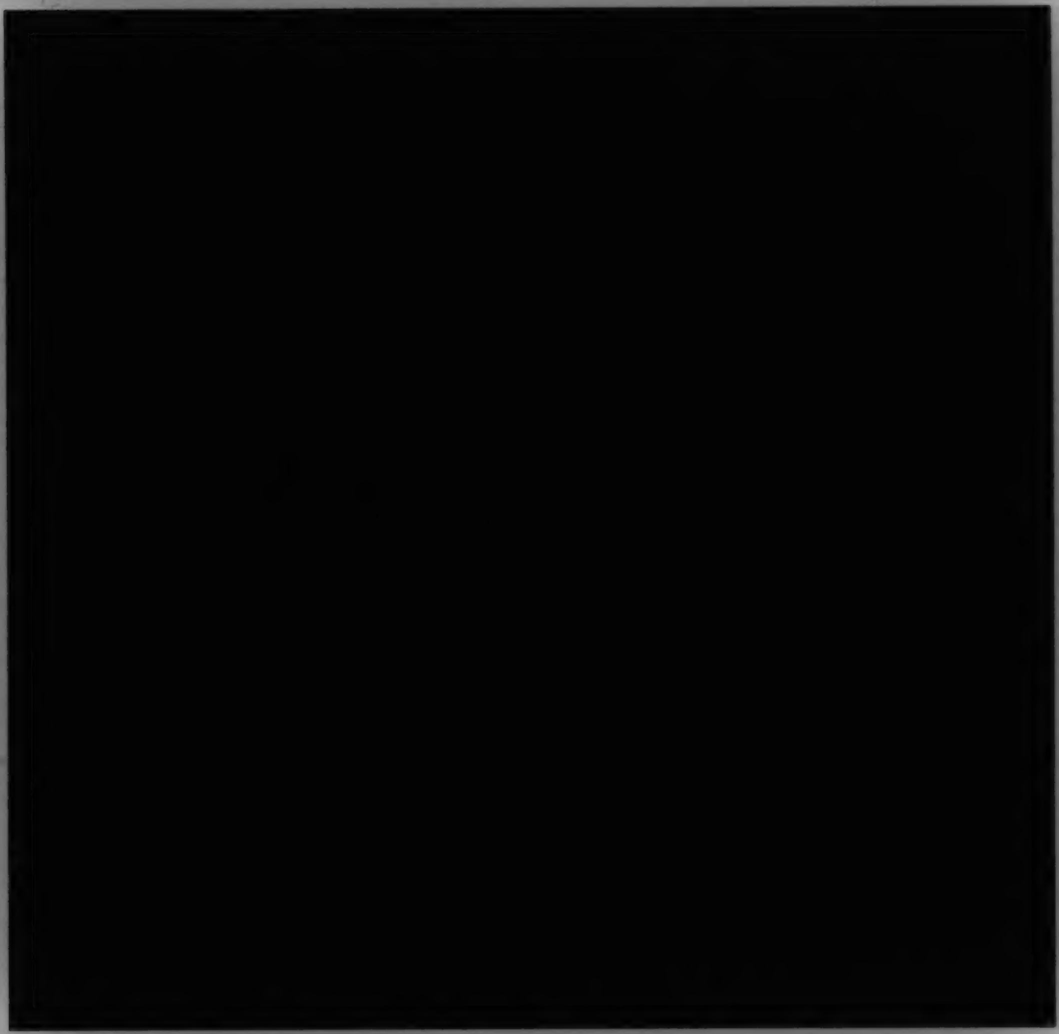
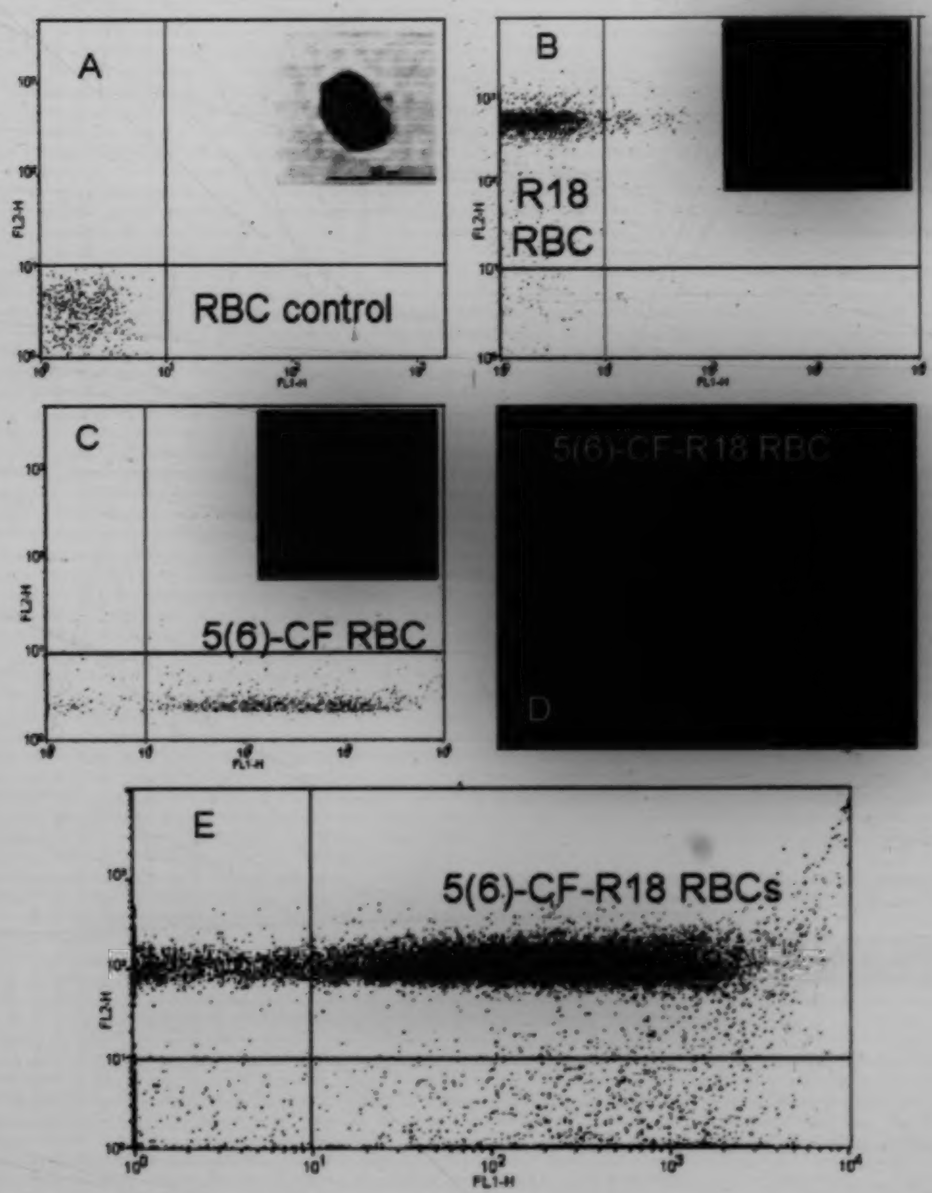


Figure 4.9. Flow cytometry and confocal microscopy images show: control RBC (Figure A), transfer of hydrophobic fluorescent marker R18 (Figure B), hydrophilic marker 5(6)-CF (Figure C) and both markers (Figures D and E) following incubation with 5(6)-CF-R18 liposomes to RBCs.



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A.2. Appendix for Section 4.2.

This appendix illustrates an example of calculations used to translate trehalose concentrations spectrophotometrically measured in RBC lysate to intracellular trehalose concentration, after RBCs were incubated with 200 mM trehalose control liposomes,

(measured trehalose concentration in RBC lysate) x (solution volume) = moles of trehalose

ex. 3.75 mM x 2.2 mL = 8.23 μmol

$$\frac{\text{moles trehalose in RBC lysate}}{\text{RBC count}} \times \frac{1}{\text{RBC volume} \times (1 - \text{osmotically inactive fraction}^{*})} =$$

= intracellular trehalose concentration

$$\text{ex. } \frac{8.23\,\mu\text{mol}}{6.99 \times 10^{11}\,\text{RBC / L}} \times \frac{1}{91 \times 10^{-15}\,\text{L} \times (1 - 0.423)} = 224\,\mu\text{M}$$

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[*] RBC osmotically inactive fraction value was obtained by averaging three values reported in the literature.[37, 38, 39]

Chapter 5*

Greasing Fusion Machinery: Experimental Parameters Affecting Liposome-RBC Interactions and RBC Membrane Quality

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*This chapter with minor modifications has been submitted for publication as: Holovati JL, Gyongyossy-Issa M and Acker JP. Greasing liposome fusion machinery for improved intracellular trehalose delivery. Cell Preservation Technology [Submitted June 2008]

5.1. Introduction

The investigations on the interactions of fluorescently-labelled trehalose-containing liposomes with human RBCs in the previous chapter have shown that the mechanism of interaction includes both liposomal fusion with the RBC membranes, as well as adsorption of the vesicles onto the RBC surface. The previous chapter also provided convincing evidence that liposomes permeabilize RBC membranes to deliver trehalose to the RBC cytosol, supporting the hypothesis that liposomes can be used as trehalose delivery vesicles. However, the fusion efficiency of liposomes evaluated in the previous chapter was low, with only micromolar concentrations of trehalose delivered to RBC cytosol. As experimental parameters have been shown to play a key role in controlling the extent of liposome uptake by mammalian cells,[1; 2; 3; 4; 5; 6] the effects of these factors on the interaction of trehalose-containing liposomes with human RBCs need to be further investigated.

The link between the extent of the liposome-cell interaction and liposome physical characteristics has been established.[2; 3; 5; 7; 8; 9] For example, in 1973 Papahadjopolous *et al.* investigated the ability of liposomes composed of a variety of natural and synthetic lipids to induce fusion to several cell lines in both suspension and monolayer cultures.[3] According to this study, liposome surface charge, both positive and negative, increases liposome fusion capacity compared to uncharged liposomes. This finding was confirmed by Martin and MacDonald, who have demonstrated that synthesizing liposomes with a composition that closely mimics the highly fusogenic paramyxovirus Sendai significantly induced liposome fusion to chicken RBCs.[6] They also concluded that the degree of fusion largely depends on the presence of charge on liposome membranes.[6] In addition to membrane charge effect, Papahadjopolous' study demonstrated that liposome membrane fluidity markedly affects the extent of liposome fusion.[3] Liposomes containing phospholipids that were below their T_m, and therefore in solid-like state with ordered hydrocarbon chains, resulted in significantly less cell fusion under the same experimental conditions compared to liposomes containing phospholipids that were either at or above their T_m, and therefore in a more fluid state with disordered hydrocarbon chains.[3] Membrane fluidity is not only affected by phospholipid T_m, but also by the presence of cholesterol, as it modifies the packing and mobility of phospholipid molecules.[4; 7; 10; 11] In addition to affecting the overall extent of liposome fusion, many authors have shown that by manipulating the membrane composition of vesicles, it is possible to achieve some control over the mechanism of liposome uptake as well as some degree of targeting of vesicles and their contents to different regions of the cell.[5; 7; 9; 12; 13; 14; 15; 16] However, in contrast to the importance of such liposome membrane parameters as surface charge and fluidity, liposome size and lamellarity do not seem to significantly affect liposome interaction with cells. Poste and Papahadjopolous were unable to detect any difference in the cellular uptake for unilamellar and multilamellar liposomes of variable sizes but identical composition.[16]

In addition to liposomal physical characteristics, liposome-cell interactions are considerably affected by experimental conditions, such as liposome to cell ratio, incubation time, temperature and solution. For example, in 1972 Magee and Miller attempted to define experimental conditions for attachment of multilamellar liposomes to ML cells in a monolayer.[17] Papahadjopolous *et al.* showed that increasing vesicle to cell ratio results in increased liposome uptake, with incorporation of up to 10^6 vesicles per cell.[2] This uptake was linear over a broad range of liposome concentrations.[2] This study also showed that the uptake of unilamellar liposomes by cultured cells is both temperature and time dependent.[2] Pagano *et al.* confirmed the importance of time and temperature for liposome uptake.[18] At 37 °C, liposomes interacted predominantly by fusion and endocytosis, while at 2 °C liposome adsorbtion to cell membrane and exchange of lipids between liposomes and cells were predominant.[18] Although different cell types vary significantly in the number of vesicles they can incorporate, the general kinetics of liposome incorporation at 37 °C include rapid uptake within the first 2-3 h, and a plateau after 3-8 h, but vary depending on cell type and liposome composition.[1; 2; 16; 18; 19] Liposome-cell fusion is inhibited under anaerobic conditions.[6] The presence of divalent cations in the incubation medium has also been shown to effectively induce interaction and fusion between negatively charged liposomes and cells.[6; 16; 20; 21; 22; 23] Liposomes containing acidic phospholipids such as PS are particularly susceptible to cation-induced fusion.[6; 15; 16; 18; 20] The fusogenic effectiveness of Ca^{2+} has been shown to be higher compared to other divalent cations, such as Mg^{2+} or Ba^{2+}.[5; 20] Incubation of liposomes in plasma or serum leads to dramatic increase in leakage of small solutes from liposome aqueous core and altered liposome-cell interaction.[24; 25; 26; 27; 28]

Liposome physical characteristics and other experimental parameters not only affect the extent, pathway and kinetics of liposome-cell interaction. Incubation of various liposomes under different experimental conditions can also have a pronounced effect on cell morphology, growth and viability.[1; 5; 15; 16; 24] For example, in 1976 Dunnick *et al.* demonstrated that incubation of EMT-6 tumor cells with liposomes results in delivery of phospholipids and cholesterol to cell membranes, but also inhibited cell growth.[29] Prolonged incubation of liposomes with murine lymphocytes led to vesicle uptake, but also resulted in significant changes in cell morphology and loss of cell viability.[22] Inclusion of fusogenic phospholipid lecithin markedly induced liposome fusion to cells, but also led to hemolysis and cytotoxicity.[6; 16; 30] RBCs incubated with liposomes containing more cholesterol than phospholipids resulted in significant cholesterol enrichment of the RBC membrane and shape change from discocyte to codocyte.[31] In contrast, Papahadjopolous *et al.* reported no significant cell injury after negatively charged liposomes were taken up by mouse fibroblasts and sheep erythrocytes.[3] Similarly, Pagano *et al.* reported no changes in viability of the ability to proliferate after liposome interaction with the hamster cell line.[18] Platelet-liposome fusion has shown not to cause overt changes to platelet function, such as aggregation or hemostasis.[32] Therefore, the adverse effects of liposome-cell interaction are highly dependant on the liposome and cell type, as well as the experimental conditions.

This thesis is investigating liposomal delivery as a permeabilization strategy for the intracellular accumulation of trehalose into human RBCs. As experimental parameters have been shown to play a significant role in liposome-cell interaction and post-interaction cell viability, this chapter investigates the effects of several experimental variables, including the composition of liposome membrane and aqueous core, as well as the incubation conditions, including liposome to cell ratio, incubation time, temperature, and medium on liposome-cell interaction, trehalose loading and RBC membrane quality. We hypothesize that manipulating liposome physical properties and incubation conditions will result in increased RBC uptake of trehalose-containing liposomes, with minimal detrimental effects on RBC membrane quality.

5.2. Materials and Methods

5.2.1. The Effects of Incubation Conditions on Liposome-RBC Fluorescence Transfer

5(6)-CF trehalose-containing liposomes, synthesized in the previous chapter to investigate modes of liposome-RBC interaction, were also used to examine the effects of liposome concentration, incubation time and temperature on fluorescence transfer to RBCs. Several concentrations of these liposomes (0.25, 0.5, 1, 2, 4, 5 and 6 mM lipid, based on the phosphate assay results described in Chapter 3, Section 2.2.1) were incubated with human RBCs, according to protocol described in Chapter 4, Section 2.2. The transfer of fluorescence from liposome to RBC population was monitored using flow cytometry, before and after density gradient separation, as described in Sections 4.2.3 and 4.2.4. To investigate the effect of incubation temperature, RBCs were incubated with 1 mM lipid of 5(6)-CF trehalose-containing liposomes for 2 h at 4 °C, RT and 37 °C, and to investigate the effect of incubation time, RBCs were incubated with 1 mM lipid of 5(6)-CF trehalose-containing liposomes at RT (21 °C) for 15 min, 1 h and 2 h. RBCs were then separated from liposomes that were adsorbed to the RBC membranes, along with the free liposomes in the incubation mixture using density gradient separation, and cellular fluorescence intensity assessed as previously described (Section 4.2.4). The results were expressed in arbitrary units of 5(6)-CF fluorescence.

5.2.2. The Effect of Liposome Aqueous Core Composition on Intracellular Trehalose Delivery

Chapter 2 describes synthesis and characterization of unilamellar liposomes containing 200, 300 and 400 mM trehalose in their aqueous core. Due to liposome membrane composition of DPPC and cholesterol, these liposomes can also be described as 'uncharged' when resuspended in a physiological buffer.[4] Concentrations of these liposomes (2, 4, and 6 mM lipid) were incubated with human RBCs for 2 h at 37 °C, then removed using density gradient separation and washing procedures. RBCs were lysed by immersing into liquid nitrogen and slow thawing at room temperature three times, as described in Section 4.2.3. Megazyme spectrophotometric method for trehalose measurement is described in detail in Chapter 3, Section 2.2.4. Intracellular trehalose concentration was calculated based on measured trehalose in lysate solution, RBC count, and RBC volume corrected for the osmotically inactive fraction, as described in Appendix A.2.

5.2.3. The Effect of Liposome Membrane Composition on Liposomal Trehalose Delivery

Negatively charged trehalose-containing liposomes were synthesized by dissolving PS (Avanti Polar Lipids, Alabaster, AL) together with DPPC and cholesterol in chloroform at a respective molar ratio of 1:6:3, resulting in a 25 mM final lipid solution. Lyophilized lipids were then hydrated with 300 mM trehalose buffer (refer to Table 3.1 for exact composition, pH and osmolality), followed by previously described steps for synthesis of unilamellar liposomes (Chapter 3). Due to the PS in their membrane, when resuspended in a physiological buffer, these liposomes can also be described as 'negatively charged'.[4] After synthesis, negatively charged liposomes were characterized for lipid content, size and trehalose content, according to assays described in Chapter 3. Concentrations of these liposomes (2, 4 and 6 mM lipid) were incubated with human RBCs at 37 °C, for incubation times ranging from 1 to 24 h. Liposomes and RBCs were incubated in either HBS buffer or 300 mM trehalose buffer (Table 3.1), each containing 5 mM of $CaCl_2$ (Sigma-Aldrich, St. Louis, MO). The uptake of trehalose by RBCs was measured spectrophotometrically, as a function of the time of incubation, liposome charge and liposome concentration, as described above. Uncharged liposomes hydrated with 300 mM trehalose (from Section 5.2.2) were also included in these studies to distinguish the effect of liposome membrane charge and presence of $CaCl_2$ on liposome trehalose delivery.

5.2.6. The Effects of Liposomes on RBCs Membrane Quality

To examine the effect of liposome permeabilization on RBC quality, percent hemolysis using the cyanmethemoglobin method, as described in Chapter 2, Section 2.2.1, was determined immediately after RBC were treated with negatively charged liposomes (4 mM liposome lipid, incubation at 37 °C for 4 h). In addition, RBC morphology was evaluated by examining unstained RBCs with light microscopy. Flow cytometry was used to more closely examine the quality of RBC membrane by focusing on PS externalization and loss of CD47 antigen, using the protocol described in Chapter 2, Section 2.2.2. RBCs were then hypothermically (1-6 °C) stored in 300 mM trehalose buffer (Table 3.1) and percent hemolysis assay repeated at 4, 24, 48 and 72 h. To parallel the investigation of the effects of different incubation conditions on trehalose loading, RBC quality post-permeabilization was evaluated as a function of the time and temperature of incubation, liposome charge and liposome concentration. In these experiments, RBC recovery was calculated by comparing RBC counts from a hematology cell analyzer (Beckman Coulter AcT, New York, NY) before and after liposome treatment, while flow cytometric analysis of PS externalization was used as a measure of RBC membrane quality. To examine the effect of incubation time, 4 mM of negatively charged liposomes were incubated with

RBCs at 37 °C for 2, 4, 24 and 48 h, while the effect of incubation temperature was investigated by incubating 4 mM of negatively charged liposomes with RBCs at 4°C, RT and 37 °C for 24 h. Percent RBC recovery was also examined after RBCs were incubated with various concentrations (2, 4 and 6 mM) of charged and uncharged liposomes at 37 °C for 4 hours.

Unless indicated otherwise, the data points presented in this chapter represent the mean values from three experiments with error bars denoting the standard deviation. RBCs maintained in HBS buffer under the same conditions with respect to incubation conditions, but without exposure to liposomes, were run in parallel for each experiment and are referred to as control RBCs.

5.3. Results

5.3.1. The Effects of Incubation Conditions on Liposome-RBC Fluorescence Transfer

The effect of liposome concentration on fluorescence transfer from liposomes containing aqueous core labelled with 5(6)-CF marker to human RBCs is shown in Figure 5.1. RBC mean fluorescence intensity increases with increasing concentrations of 5(6)-CF liposomes following a 2 h RT incubation, suggesting a strong positive correlation between liposome lipid concentration and mean cellular fluorescence intensity. Increase in liposome concentration resulted in continuous increase in RBC mean fluorescence intensity if liposome-RBC incubation mixture was analyzed on a flow cytometry without the density gradient separation, with a sharp increase between 4 and 6 mM liposome lipid (Figure

5.1.A). RBC mean fluorescence intensity also increased with increase in liposome lipid concentration after the density gradient separation, but this effect plateau at higher liposome concentrations (4 – 6 mM lipid, Figure 5.1.B).

In addition, there is a strong positive correlation between RBC mean fluorescence intensity and incubation time and temperature after the density gradient separation, as depicted in Figure 5.2. Figure 5.2.A. is the graphical representation of the temporal response. The increase in fluorescence intensity with increase in incubation time is seen as a right shift in the fluorescence histogram of gated RBC population. There was a 447 ± 37 % increase in the RBC FL1 fluorescence after 15 min incubation with 5(6)-CF liposomes, compared to the autofluorescence intensity of control RBCs. Additional 45 min increase in incubation time resulted in additional 427 ± 44 % increase in RBC fluorescence intensity, while prolonging incubation from one to two hours increased RBC mean fluorescence intensity by 172 ± 32 %. Figure 5.2.B indicates that cell-liposome interaction is also temperature dependent. RBCs exposed to 5(6)-CF liposomes for 2 h at 37 °C had a fluorescence intensity 163 ± 12 % greater than RBCs incubated with 5(6)-CF liposomes for 2 h at RT, while there was no marked increase in mean fluorescence intensity of RBCs incubated with liposomes at 4 °C compared to the autofluorescence intensity of control RBCs (Figure 5.2.B).

5.3.2. The Effects of Liposome Composition on Liposome Trehalose Delivery

The effect of liposome aqueous core composition on intracellular trehalose loading is illustrated in Figure 5.3. A two-hour, 37 °C incubation of RBCs with liposomes containing 300 mM trehalose in their aqueous core resulted in increased intracellular trehalose delivery compared to RBCs incubated with liposomes containing 200 and 400 mM trehalose in their aqueous core. This effect was seen with three different concentrations of liposomes (2, 4 and 6 mM lipid). In addition to liposome aqueous core composition, liposome membrane composition / charge had a major effect on intracellular trehalose loading, as illustrated in Figure 5.4. Liposome characterization assays showed that negatively charged liposomes had similar physical characteristics to uncharged liposomes (mean liposome lipid concentration 22.9 ± 1.8 mM, mean liposome size 397 ± 6 nm, and mean intra-liposome trehalose concentration 180.4 ± 8.9 mM, n=3). However, negatively charged liposomes containing PS in their membrane delivered about 100 times more trehalose than neutral liposomes with only DPPC and cholesterol in their membranes under the same incubation conditions (Figure 5.4). Increased trehalose delivery of negatively charged liposomes was evident at different concentrations of liposomes (Figure 5.4), different incubation times (Figure 5.5) and in different incubation buffers (Figure 5.6).

5.3.3. The Effects of Incubation Conditions on Liposome Trehalose Delivery

Similar to fluorescence transfer, incubating human RBCs with increasing concentrations of liposomes resulted in increased intracellular trehalose delivery (Figure 5.4). For example, incubating RBCs with 4 mM negatively charged liposomes for 4 h at 37 °C resulted in 59 ± 6 % more intracellular trehalose than incubating RBCs with 2 mM negatively charged liposomes under the same incubation conditions. However, the increase in intracellular trehalose delivery between 4 mM and 6 mM negatively charged liposomes was only 6 ± 2 %, which parallels the plateau seen with 5(6)-CF fluorescence transfer at higher liposome concentrations in Figure 5.1.B. In addition, as illustrated in Figure 5.5, prolonged incubation leads to increase in intracellular trehalose delivery, which was highest after 24 h, 37 °C incubation with negatively charged liposomes. Finally, RBC-liposomes incubation in trehalose extracellular solution, as opposed to buffered saline, lead to 34 ± 4 % increase in RBC intracellular trehalose concentration (Figure 5.6).

5.3.4. The Effects of Liposomes on RBCs Membrane Quality

The effect of liposome treatment on percent hemolysis immediately after the liposome treatment, and after subsequent hypothermic storage in trehalose buffer is illustrated in Figure 5.7. There were not marked differences in percent hemolysis between the control and liposome treated RBCs, suggesting that liposome trehalose delivery does not cause significant injury to RBC membranes. Coulter analysis showed that there ware no marked differences in RBC MCV between liposome treated and control RBCs, indicating that liposome treatment did not result in detectable changes to RBC size. Furthermore, RBC treatment with negatively charged liposomes (4 mM liposome lipid, incubation at 37 °C for 4 h), which resulted in intracellular delivery of 15.3 mM trehalose did not adverlsy affects on RBC morphology, as shown in Figure 5.8. This figure also shows that such liposome treatment did not result in more subtle RBC membrane lesions, as there was no increase in annexin V binding to exposed PS on RBC membrane surface and no visible changes in the expression of CD47 antigen (Figure 5.8). However, prolonged RBC - liposome incubation and higher incubation temperatures resulted in increased cell injury, as illustrated by increased RBC loss and membrane PS exposure in Figure 5.9. The effects of liposome concentration and charge on RBC recovery after RBCs were incubated with 2, 4 and 6 mM of charged and uncharged liposomes at 37 °C for 4 hours is shown in Figure 5.10. RBC recovery was 84 ± 5 %, regardless of liposome concentration or membrane charge. These results show that, in contrast to incubation time and temperature, liposome concentration and charge are not important predictors of RBC injury.

5.4. Discussion

According to Poste *et al.*, mammalian cells "display a remarkable appetite for lipid vesicles".[19] Although the previous chapter provided convincing evidence that RBCs incorporate trehalose-containing liposomes, the RBC appetite for these liposomes has been shown to be far less than remarkable, with only micromolar concentrations of trehalose delivered to the RBC cytosol (Chapter 4, Table 4.2). The results of this study demonstrate that by modifying the composition of the liposome aqueous core and lipid bilayer, as well as experimental conditions such as liposome to cell ratio, incubation time, temperature and medium on liposome-cell interaction, the RBC appetite for trehalose-containing liposomes can be increased without major adverse effects on RBC membrane quality.

Extensive research on liposome-cell interactions has shown that one single type of liposome formulation can interact with different cell types to different degrees. Similarly, different formulations of liposomes vary in their extent of interactions with the same cell type. As this thesis focuses on mature RBCs, cell type remained constant and we varied the physical properties of liposomes. Increasing the trehalose concentration of the liposome aqueous core somewhat improved liposome trehalose delivery. However, this increase in delivery approach does not even remotely to the extent of the dramatic change in liposome uptake caused by including acidic phospholipid PS into the liposome membranes. Negatively charged liposomes containing PS in their membranes delivered approximately 100 times more trehalose to the RBC cytosol compared to uncharged liposomes under the same conditions (Figure 5.4), which confirms previous research suggesting that liposome membrane charge is of major importance in the incorporation of vesicles into mammalian cells.[6; 16; 20; 21; 22; 23; 33]

At first sight, it appears surprising that negatively charged liposomes are able to get in close proximity to the highly negatively charged surface of mammalian cells, which is necessary for fusion to occur. Consequently, several mechanisms have been proposed to explain the enhanced fusion of negatively charged liposomes. One of the earlier theories suggests that although the overall mammalian cell surface charge is negative, the distribution of negatively charged groups on the mammalian cell surface is not homogeneous, so the liposome-cell contact occurs preferentially at areas with low negative surface charge through electrostatic interaction. [34; 35; 36] In addition, several theories have focused on the role of the divalent cations in the fusion of negatively charged liposomes. One hypothesis is that Ca^{2+} binds anionic phospholipid membranes, neutralizing the negative charge on both liposome and cell membranes and thus facilitating aggregation.[20; 37; 38] Also, it has been shown that cations in the solution induce morphological changes in PS-containing liposomes by interacting with carboxyl groups on phospholipid molecules and/or with membrane fusion-inducing SNARE proteins, resulting in phospholipid aggregation within the membrane.[33; 39; 40] Phospholipid aggregation leads to packing irregularities that may cause membrane structure instability favoring further aggregation and fusion.[33; 39; 40] An alternative explanation for the cation fusion promotion is based on the tendency toward dehydrated complexes. Ca^{2+} is thought to alter the membrane hydrophobicity both by replacing the water of hydration and by exposing the hydrocarbon interior of the membrane, subsequently leading to membrane fusion.[18; 20; 34] Finally, divalent cation-induced fusion has been shown to be accompanied by a change in the phase behaviour of membranes, so this ability to shift the gel-to-liquid crystalline phase transition temperature has been correlated with cation fusogenic capacity.[41; 42] Regardless of the specific mechanism(s), our results demonstrated that cation-induced fusion of negatively charged trehalose-containing liposomes result in a significant increase in liposome-RBC interaction and intracellular trehalose delivery (Figure 5.4), without major injury to RBC membranes (Figure 5.9).

Increasing the intra-liposomal trehalose concentration from 200 to 300 mM resulted in increased RBC trehalose delivery, but this effect surprisingly was not seen when intra-liposomal trehalose concentration was increased from 300 to 400 mM. The osmolality of the incubation buffer may play a role in the reduced uptake of 400 mM trehalose liposomes. For successful liposome synthesis, it is strongly advised that the hydrating buffer for the liposome aqueous core have a similar osmolality to that of the resuspending buffer.[4; 10; 26; 43] Trehalose liposomes containing 400 mM trehalose in their aqueous core were therefore resuspended in 394 mOsm HBS buffer (Table 3.1). Although this buffer is isoosmotic for liposomes, it is hypertonic for RBCs, and results in the efflux of water out of the cell, leading to RBC shrinkage. We therefore hypothesize that this change in RBC shape may negatively affect how RBCs interact with liposomes and, consequently, the extent of liposome-RBC fusion. The effects of osmolality on liposome-cell fusion warrant further studies.

In addition to liposomal physical characteristics, interactions between trehalose-containing liposomes and human RBCs were considerably affected by experimental conditions. As expected, RBC uptake of trehalose-liposomes was temperature sensitive (Figure 5.2.B). Increased liposome uptake may be attributed to higher liposome and RBC membrane fluidity at increased incubation temperatures. At 37 °C, RBC membranes are in a liquid-crystalline state [44] and are susceptible to the formation of sphingolipid membrane rafts,[45] which represent favourable conditions to liposome-cell aggregation and fusion.[1; 20; 46] Higher trehalose delivery when RBCs and liposomes were incubated in trehalose buffer as opposed to saline buffer (Figure 5.6) can be explained by a probable passive trehalose influx from extracellular medium during transient membrane disruptions upon liposome fusion. In addition, the RBC membrane has been shown to be somewhat permeable to trehalose at 37 °C.[47] The time- and concentration-sensitive nature of liposome uptake with more extensive initial delivery that eventually plateaus (Figures 5.1.B, 5.2.A, 5.3. and 5.4) correlates well with previously published data.[1; 2; 3; 18; 29] The lack of a plateau in Figure 5.1.A illustrating the continuous increase in RBC fluorescence intensity with increased 5(6)-CF liposome concentration, when RBC-liposome incubation mixture was analyzed without the density gradient. This supports the findings from the previous chapter that liposome coating of RBC membranes plays a significant role in liposome-RBC interaction.

Although a prolonged incubation of 24 h at 37 °C resulted in a marked increase in intracellular trehalose loading (Figure 5.5), it also resulted in injury to

RBC membranes (Figure 5.9). Our results suggested that, in contrast to incubation time and temperature, liposome concentration and charge are not important predictors of RBC injury (Figure 5.8 and 5.9). Therefore, improving liposome trehalose delivery involves balancing the efficiency of trehalose uptake with the minimization of cell membrane injury resulting from prolonged liposome-cell incubation at higher temperatures. However, it is important to emphasize that our results showed that liposome treatment and trehalose loading did not cause significant adverse effects on RBC membrane quality (Figures 5.7 and 5.8). Other proposed membrane permeabilization and trehalose loading techniques, such as introduction of a genetically engineered mutant of a pore protein,[48; 49] thermal poration,[50; 51; 52] microinjection,[53] and electroporation,[54] have all been associated with cell loss and/or membrane injury. For example, a thermo-osmotic trehalose loading method involves incubating RBCs at 37 °C in high concentrations of trehalose for prolonged periods of time, so the loading is contributed to the combination of osmotic imbalance and the phospholipid phase transition.[47; 52] Satpathy *et al.* have shown that incubating RBCs in 0.8 M trehalose at 37 °C for 7 h results in about 30 mM intracellular trehalose, but accompanied by over 20 % hemolysis.[47] Quan *et al.* have confirmed these findings, with about 20 mM intracellular trehalose loaded after 5 h incubation in 0.8 M trehalose at 37 °C.[52] However, more than 40 % of trehalose-loaded RBCs had PS exposed on their membranes.[52] In contrast, RBC incubation with negatively charged trehalose-containing liposomes (4 mM lipid, 4 h, 37 °C, in 0.3 M trehalose buffer) resulted in about 15 mM intracellular trehalose (Figure 5.6), with about 0.1 % hemolysis (Figure 5.7), and minimal adverse effects to RBC morphology, PS exposure and CD47 expression (Figure 5.8).

There are many other potential ways to further improve RBC uptake of trehalose-containing liposomes. For example, addition of a dehydrating polymer, such as polyethylene glycol (PEG) in the liposome-cell incubation medium has been shown to improve liposome delivery efficiency [55; 56; 57; 58]. PEG creates an osmotic imbalance in the external environment of membrane vesicles, inducing close contact between membranes that leads to membrane dehydration and liposome fusion [55; 57; 58]. In addition, liposome fusion can be induced by the inclusion of a low concentration of non-bilayer-forming lipids in the liposome bilayer, such as lysophosphatidylcholine [56; 59; 60]. The conical structure and intrinsic curvature of lysophosphatidylcholine predisposes target membranes to local fluctuations away from a bilayer structure, and this promotes fusion [59; 60]. However, rather than focusing on investigating the approaches to further "grease" the fusion machinery, the further studies in this thesis will assess the responses of liposome-treated RBCs to freezing.

5.5. Conclusion

Investigating the effects of various experimental parameters on liposome-RBC interaction and RBC membrane quality is an important step towards establishing liposomes as tools for the intracellular delivery of stabilizing disaccharides for biopreservation purposes. The results from this thesis chapter show that manipulating liposome physical properties, such as liposome membrane and aqueous core composition, and incubation conditions, including liposome to cell ratio, incubation time, temperature and medium, results in an increased RBC uptake of trehalose-containing liposomes, with minimal detrimental effects on RBC membrane quality. Studies in the next chapter will investigate the effects of liposome treatment on the RBC response to freezing by examining membrane post-thaw quality using both conventional and novel markers of RBC membrane injury.

Figure 5.1. The effect of liposome concentration on fluorescence transfer from 5(6)-CF labelled liposomes to RBCs before Percoll density gradient separation (Figure A) and after Percoll density gradient separation (Figure B).

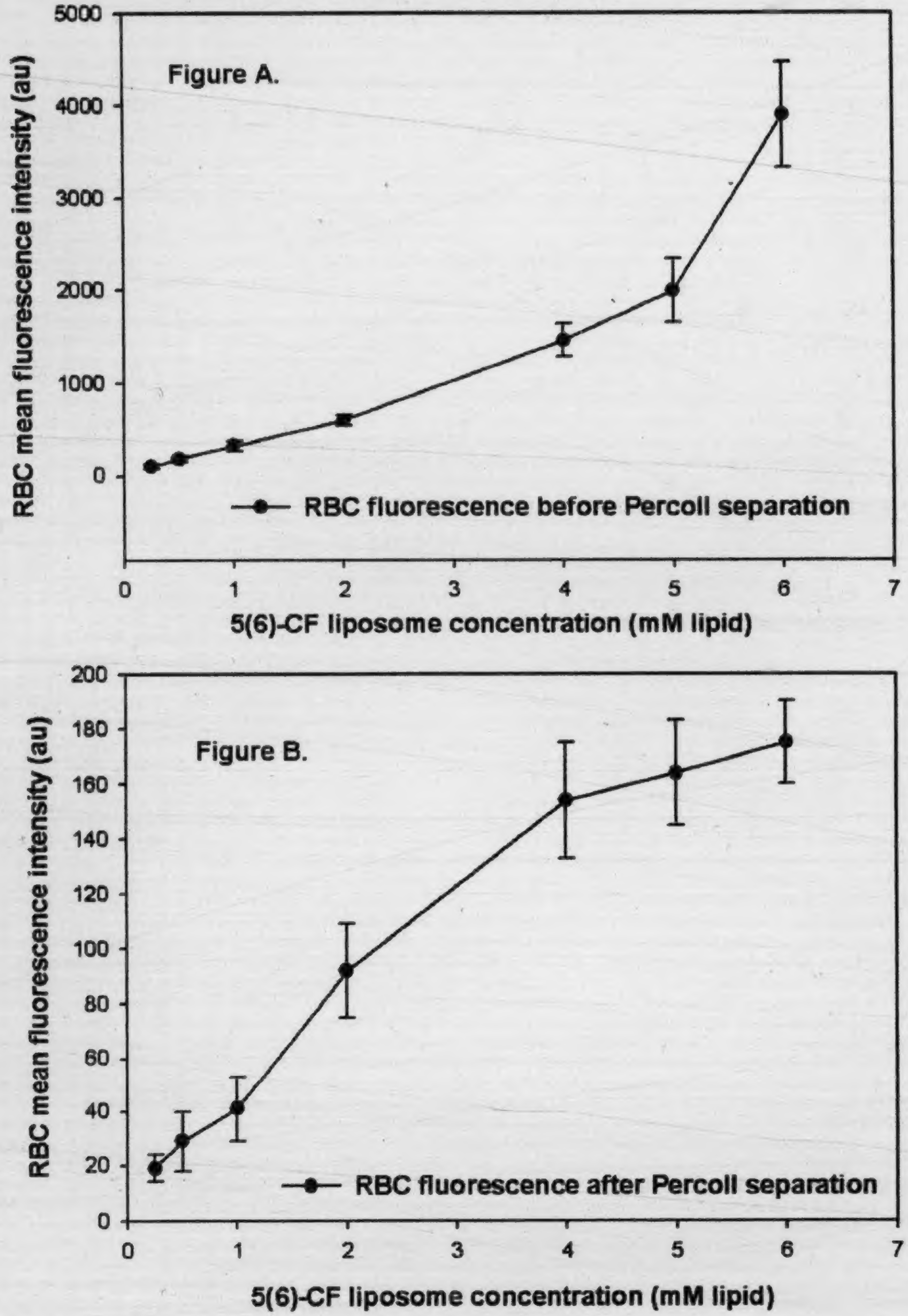


Figure 5.2. The effect of incubation time (Figure A) and incubation temperature (Figure B) on fluorescence transfer from 5(6)-CF labelled liposomes to RBCs.

Figure A

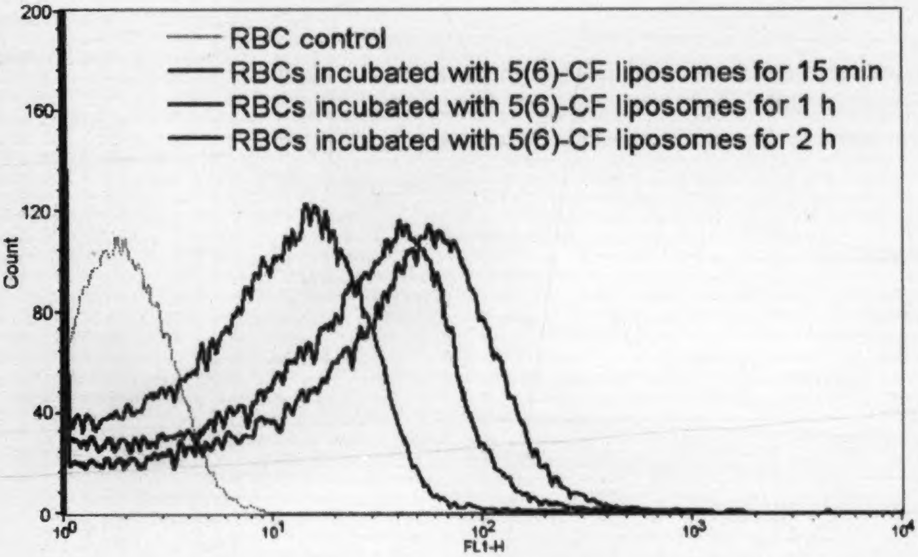


Figure B

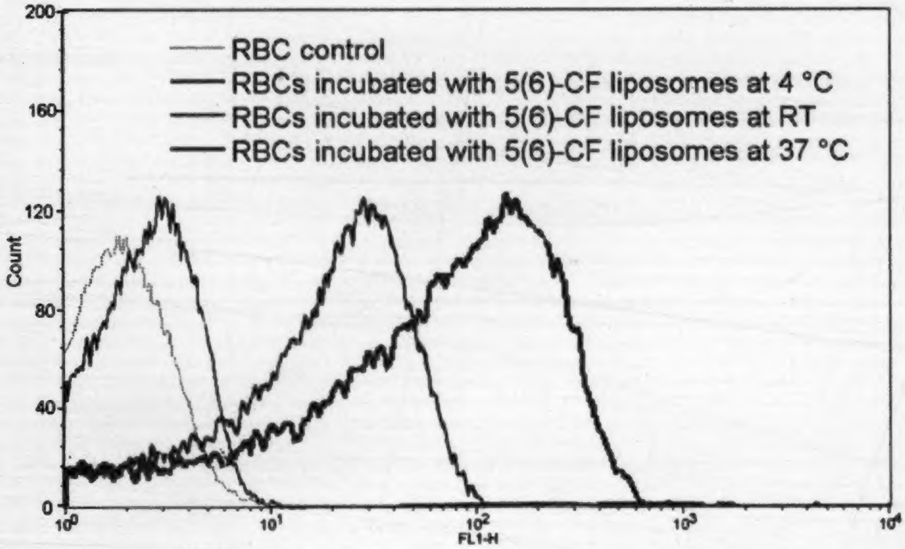


Figure 5.3. The effect of liposome aqueous core composition and liposome concentration on intracellular trehalose loading. Concentrations of the uncharged liposomes (2, 4 and 6 mM lipid) containing 200, 300 and 400 mM trehalose in their aqueous core were incubated with human RBCs for 2 h at 37 °C.

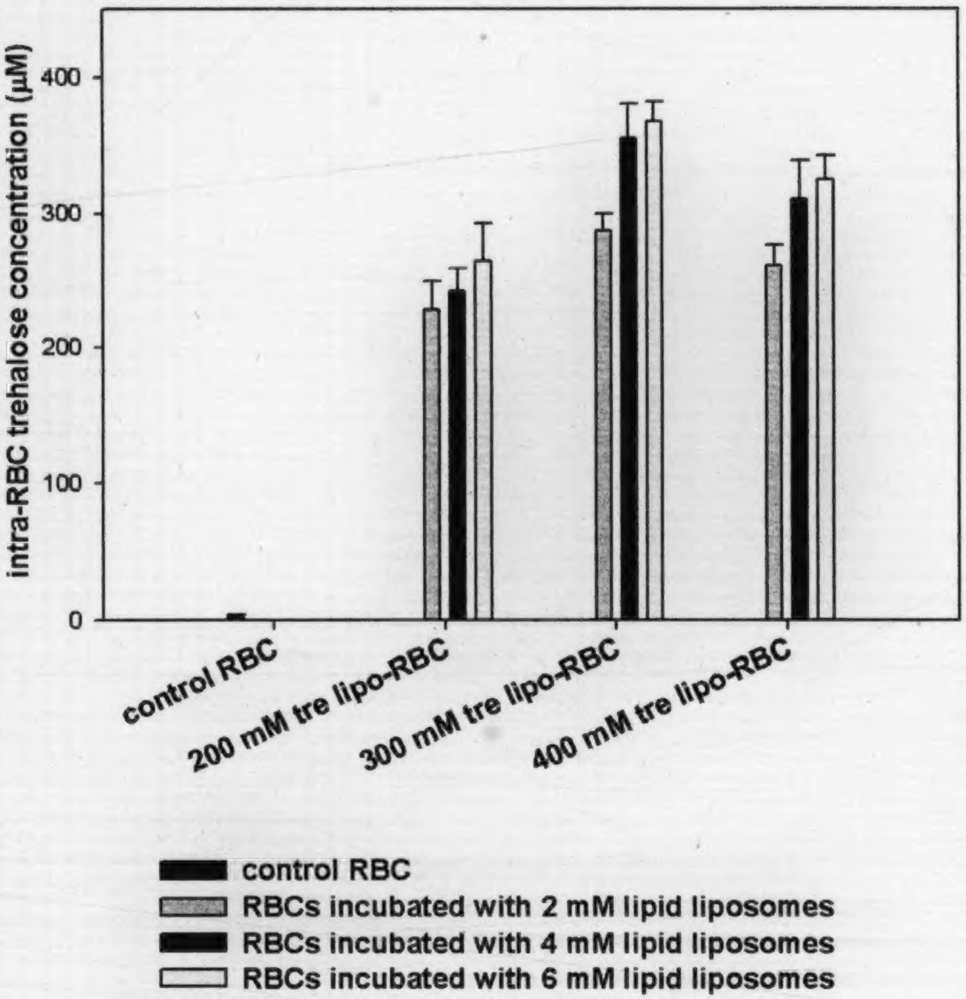


Figure 5.4. The effects of liposome concentration and charge on intracellular trehalose loading. Concentrations of the charged and uncharged liposomes (2, 4 and 6 mM lipid) were incubated with human RBCs for 4 h at 37 °C.

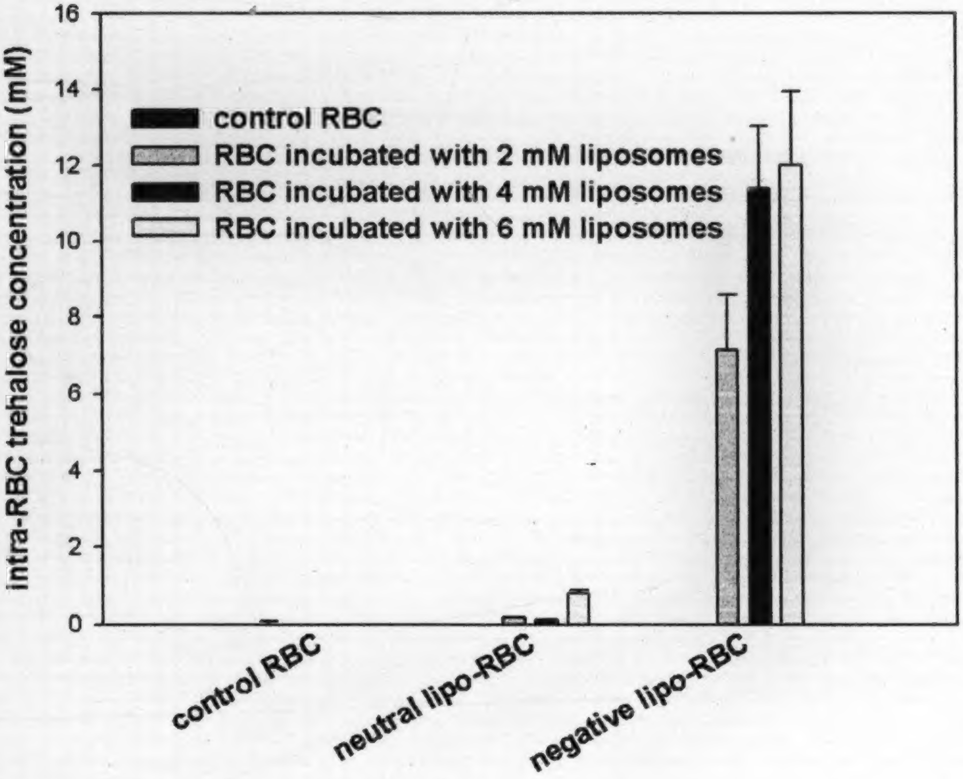


Figure 5.5. The effects of incubation time and liposome charge on intracellular trehalose loading. Charged and uncharged liposomes (4 mM lipid) were incubated with human RBCs for 1, 4 and 24 h at 37 °C.

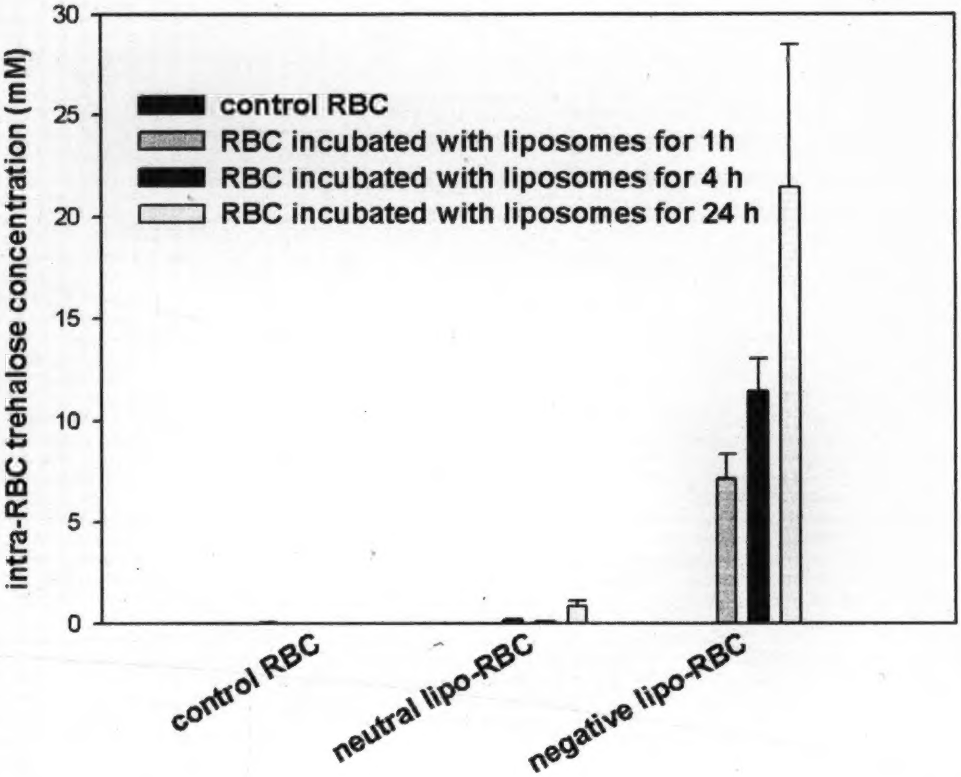


Figure 5.6. The effect of extracellular solution on intracellular trehalose loading after 4 h incubation with 4 mM liposomes at 37 °C.

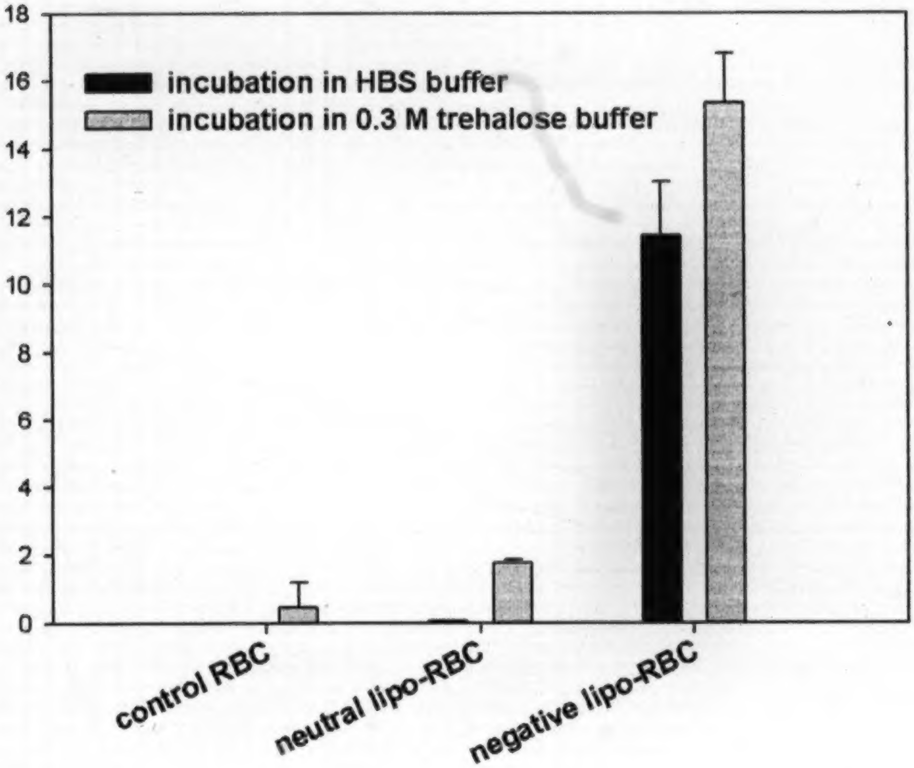


Figure 5.7. The effect of liposome treatment and subsequent hypothermic storage on percent hemolysis

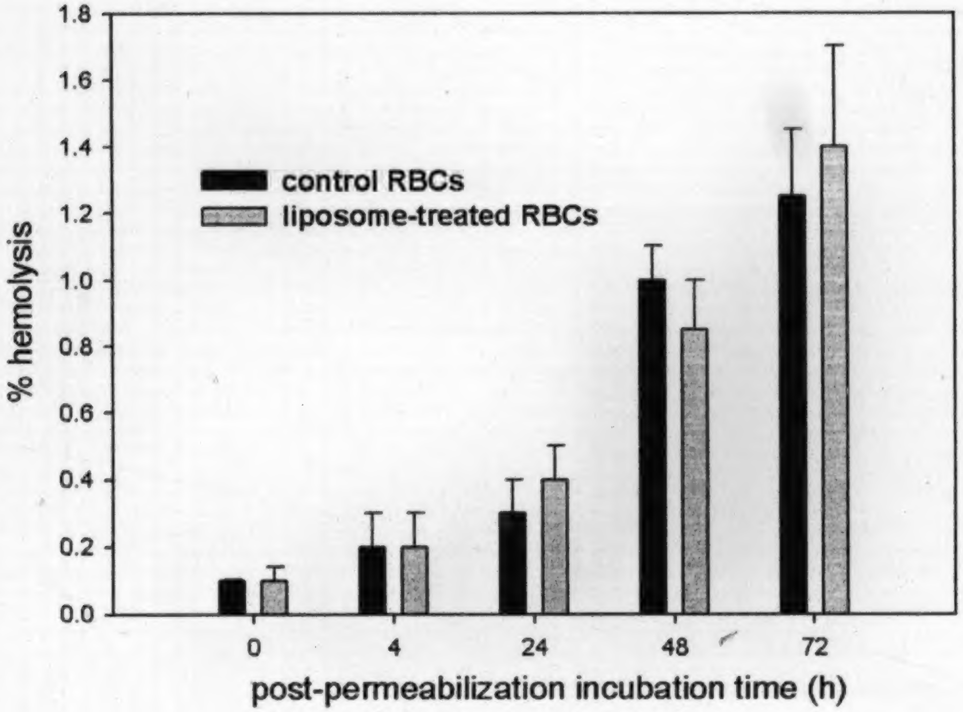


Figure 5.8. The effect of liposome treatment on RBC morphology, PS exposure and CD47 expression

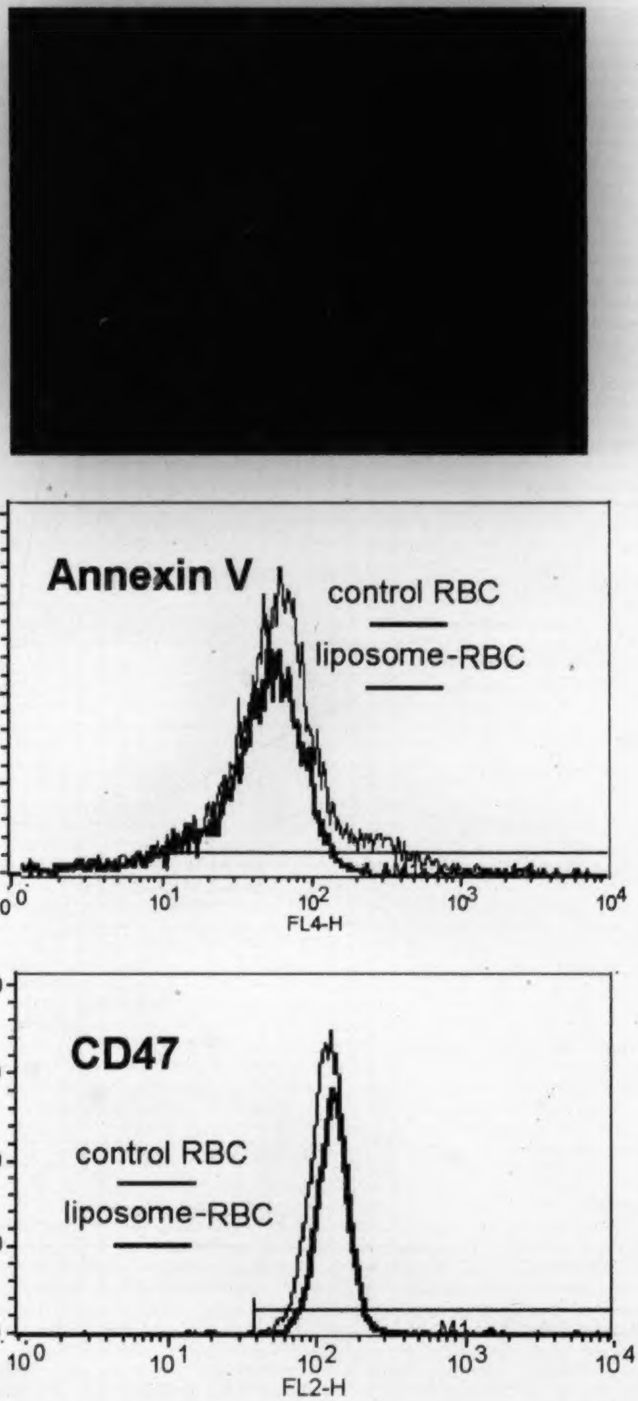


Figure 5.9. To examine the effect of incubation time on PS exposure, 4 mM of negatively charged liposomes were incubated with RBCs at 37 °C for 2, 4, 24 and 48 h (Figure A) while the effect of incubation temperature on PS exposure was investigated by incubating 4 mM of negatively charged liposomes with RBCs at 4°C, RT and 37 °C for 24 h (Figure B).

Figure A

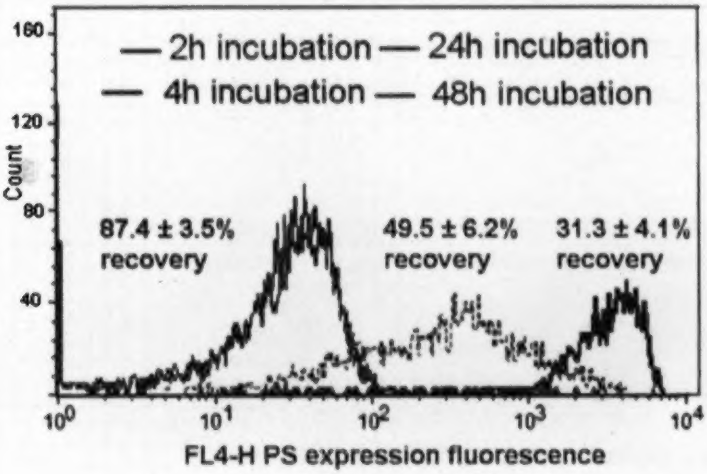


Figure B

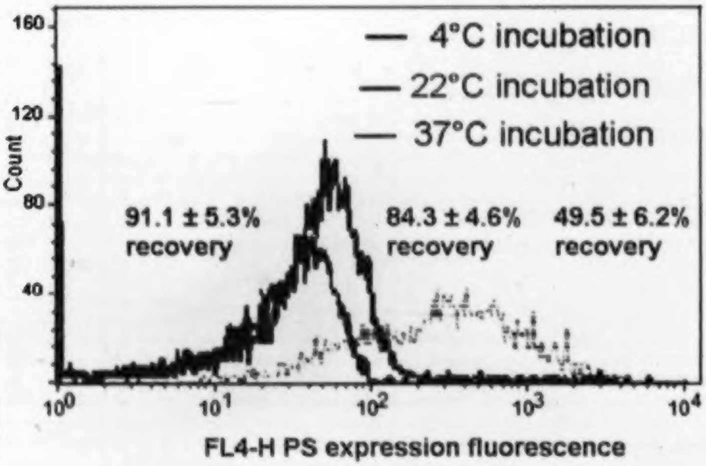
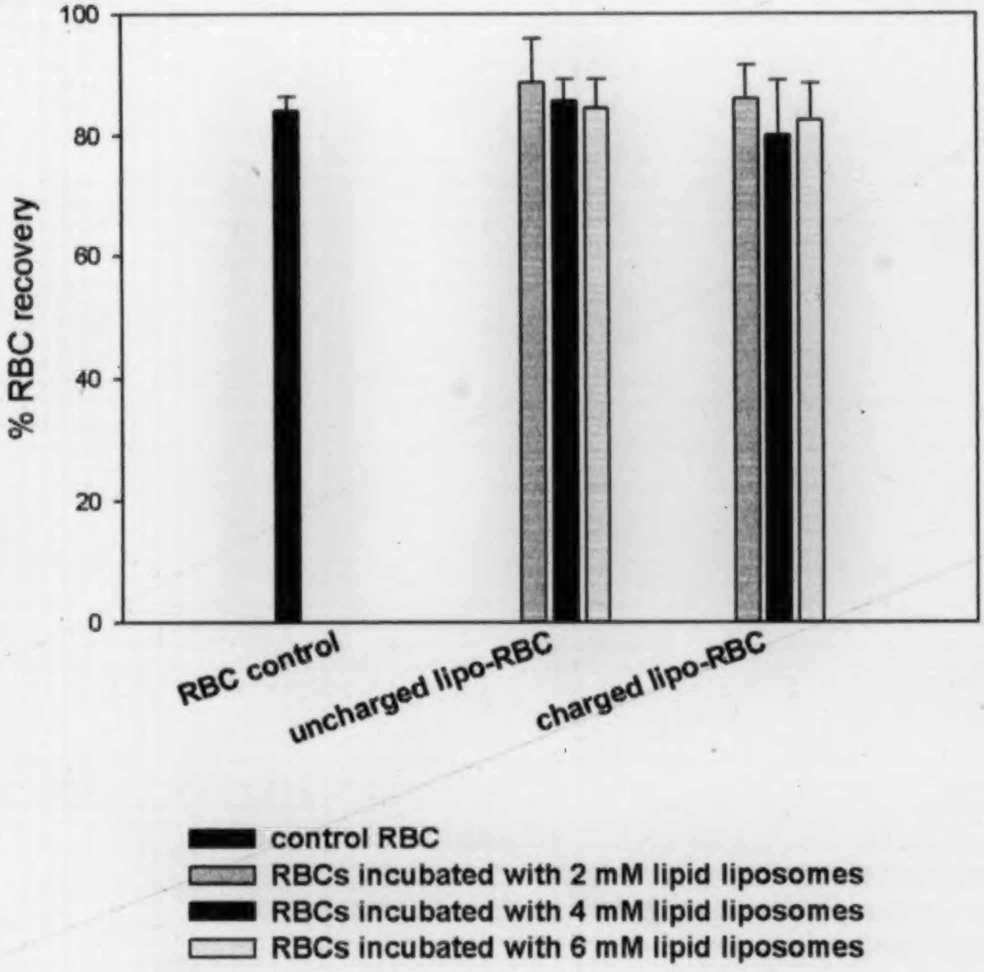


Figure 5.10. The effect of liposome concentration and charge on RBC recovery



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Chapter 6*

Effects of Trehalose-loaded Liposomes on RBC Response to Freezing and Post-thaw Membrane Quality

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6.1. Introduction

Chapter 1 summarized the basic principle of cryobiology: when cells are exposed to the ice formation that accompanies low temperatures, they are subjected to major changes in their physical environment, resulting in cell injury. Cryoprotective agents (CPAs) can be added to a cell suspension to mitigate cryoinjury and increase cell survival following freezing and thawing. The labour-intensive, technically-demanding nature of the conventional RBC cryopreservation procedures which uses glycerol as a cryoprotectant, provides the rationale for further research into the development of novel cryopreservation approaches. Another approach to RBC cryopreservation involves the use of nonpermeating CPAs, such as HES. However, nonpermeating CPAs have been shown to result in delayed hemolysis, occurring after the RBCs are transfused, and are not licensed for clinical transfusion.[1; 2; 3]

Studies of natural systems that survive extreme environmental stress, such as freezing and desiccation, have shown that one of the adaptive mechanisms is the accumulation of sugars, such as trehalose.[4; 5; 6] The mechanism of trehalose protection is an active area of research that includes the interaction of sugars with plasma membranes, [7] the role of glassy state,[8; 9] the effects on cell osmotic responses,[10; 11] and the unique physico-chemical properties of trehalose.[12; 13] Regardless of the mechanism of action, relatively low concentrations of non-toxic trehalose have been reported to act as effective CPA in a wide variety of biological systems.[14; 15; 16; 17; 18; 19] The application of trehalose as a nonpermeating intracellular CPA for RBC cryopreservation would circumvent the necessity for the expensive and tedious deglycerolization procedure, resulting in cryopreserved RBCs available for transfusion immediately upon request. However, for maximum protection efficiency, the presence of extracellular trehalose is not sufficient; trehalose presence is required on both sides of cell membrane.[14; 15; 16; 17; 18; 19] The previous chapters of this thesis have focused on investigating liposomes as delivery vesicles to introduce intracellular trehalose. The results of those studies provided evidence that, under specific experimental conditions, trehalose-containing liposomes can fuse with RBCs to deliver micromolar concentrations of trehalose to the cell cytosol, with minimal detrimental effects on RBC membrane quality. This study will investigate the effects of trehalose-containing liposomes on the RBC response to freezing.

In addition to the well-described biostabilizing properties of trehalose, liposomes themselves have been shown to protect cells under stress conditions, other than freezing. Recently, Kheirolomoom *et al.* have demonstrated that membrane lesion caused by RBC lyophilization can be reduced by adding liposomes to the lyophilization buffer.[20] During lyophilization and rehydration, RBC membranes lose phospholipids through microvesiculation, resulting in the deterioration of RBC membrane composition, morphology, and rheology.[20; 21; 22] Small unilamellar liposomes added to the lyophilisation buffer preserved membrane integrity and reduced hemolysis of lyophilized RBCs.[20] Similar RBC membrane damage has been reported by Yamaguchi *et al.*, when RBCs were subjected to high-pressure and hypotonic conditions.[23] This study also showed that the addition of aminophospholipid vesicles, in combination with amphiphatic drugs and hypertonic conditions, markedly reduced pressure-induced RBC membrane damage.[23] Liposomes were also used to reduce the chilling injury of bovine spermatozoa and oocytes.[24] As stress conditions are also associated with ice formation that accompanies low temperatures, the liposome incorporation into RBC membranes may provide an effective approach for reducing cell cryoinjury.

Trehalose and liposomes may play an important role in RBC response to freezing, but an additional major factor in determining whether or not cells survive freezing is the rate at which they are cooled.[25] As mentioned in the introductory chapter, plots of cell survival as a function of cooling rate usually exhibit an inverted U shape, with low cell survival at low and high cooling rates and higher survival at some intermediate rate (Figure 6.1). The interpretation of this curve was offered by Mazur *et al.* in his two-factor hypothesis, which remains a dominant theory in cryobiology: at low cooling rates, the cryoinjury results from exposure to concentrated extra- and intracellular solutions at high sub-zero temperatures (solution effects injury); while at high cooling rates, the cell injury is associated with intracellular ice formation (IIF).[26] The inverted U survival curve has been demonstrated for many cell types, including human erythrocytes[27], hamster tissue culture cells[26], mouse stem cells[28], human fibroblasts[29], and yeast.[30] However, although the inverted U survival curve is common for all these cell types, the optimum cooling rates vary broadly. For example, mouse stem cells show maximum survival if cooled around 1 °C/min

[28], hamster cells around 100 °C/min [26], and human RBCs over 1000 °C/min.[27; 31] Whether a given cooling rate is too high or low for a given cell type largely depends on the water permeability of the cell's plasma membrane and cell surface area to volume ratio, which are cell-type dependent properties.[26] An additional parameter affecting the relationship between the cooling rate and cell survival is the composition of the extracellular freezing solution.[27; 28; 30; 32] For example, addition of larger molecules such as sugars, starches and polymers to extracellular freezing solution will broaden the range of optimal cooling rates and reduce cryoinjury.[33; 34] It is therefore important to address the roles of cooling rate and extracellular solution when examining the effects of liposome treatment on the RBC response to freezing.

As described in Chapter 2, RBC post-thaw quality is traditionally assessed by percent hemolysis, as this is the only measure of *in vitro* quality stipulated by current regulatory bodies in transfusion medicine.[35; 36; 37] Strong evidence has recently indicated that RBC viability, defined by post-transfusion RBC survival, is closely related to the structural and metabolic status of the RBC membrane.[38; 39; 40; 41; 42] More subtle membrane changes, such as PS exposure from randomization of the asymmetric distribution of the RBC membrane and loss of CD47 antigen expression, have been shown to induce post-transfusion erythrophagocytosis, and therefore act as potentially significant predictors of RBC *in vivo* survival and function.[40; 43; 44; 45; 46; 47] Chapter 2 investigated the quality of conventionally cryopreserved RBCs using novel indicators of RBC membrane lesion and correlated them to traditional markers of

RBC injury. A similar approach will be taken in this chapter. In addition to percent hemolysis, the novel markers of subtle membrane injury will be included in evaluating post-thaw quality of liposome-treated RBCs.

The purpose of this chapter is to assess the effects of liposome-delivered trehalose on RBC post-thaw survival and quality using conventional and novel markers of RBC membrane injury. This chapter will address several key questions, including: does liposome treatment improve RBC recovery after freezing; what is the effect of different extracellular solutions and cooling rates on liposome-treated RBC response to freezing, are the effects seen due to trehalose or membrane phospholipid delivery resulting from liposome fusion; and what are the effects of freezing on liposome-treated RBC membrane quality?

6.2. Materials and Methods

6.2.1. Liposomes, Lipids and RBCs

Unilamellar trehalose-containing liposomes were synthesized using an extrusion procedure, as previously described in Chapter 3. Negatively charged, PS-containing liposomes were synthesized, according to the protocol described in Chapter 5, Section 2.3. After lyophilisation, DPPC:PS:cholesterol (6:1:3 mol %) lipid film was either hydrated with 300 mM trehalose buffer or HBS buffer devoid of trehalose (refer to Table 3.1 for exact buffer composition, pH and osmolality). Therefore, negatively charged liposomes either contained trehalose or saline in their aqueous cores. In addition to liposome synthesis, negatively charged lipids were suspended in trehalose solution without further liposome synthesis steps. To evaluate the effect of liposome charge on RBC post-thaw recovery, uncharged liposomes synthesized in the previous chapter to contain DPPC and cholesterol in their membrane and 0.3 M trehalose in the aqueous core were also used in these experiments.

RBC suspensions were prepared from phlebotomized whole blood samples from three volunteer donors, as described in Chapter 4, Section 2.2. Incubation conditions that resulted in the highest liposome uptake and minimal RBC membrane injury were chosen according to the results from the previous chapter (Section 5.3). RBCs were incubated with either charged/uncharged liposomes or lipids (4 mM) in 300 mM trehalose buffer, at 37 °C for 4 h on a laboratory shaker with low speed setting (VWR International, West Chester, PA). After incubation, density gradient separation was used to separate RBCs from unbound liposomes that were adsorbed to the RBC membranes, along with the free liposomes in the incubation mixture (Chapter 4, Section 2.3). After the separation, the supernatant containing liposome layer was aspirated, and the RBC pellet was washed with HBS buffer (Table 3.1). Liposome-treated RBCs (l-RBCs) were counted on a hematology cell analyzer (Beckman Coulter AcT, New York, NY), and diluted to an appropriate count in one of the three freezing solutions. Table 6.1 describes abbreviations used to describe different liposome preparations and treatments of RBCs.

6.2.2. *Freezing Experiments*

Three different freezing solutions were used in these studies. After density gradient separation, I-RBCs were either resuspended in 0.3 M trehalose buffer, HBS buffer (both are the same as liposome hydration buffers, Table 3.1) or in HBS buffer containing negatively charged trehalose-containing liposomes (4mM lipid). These I-RBC suspensions (500 µL) were transferred to 1.5 mL polypropylene cryo-tubes, which were then immersed in a methanol bath (Multi-Cool, FTS Systems Inc., Stone Ridge, NY) and cooled to -5 °C. A thermocouple (30 gauge wire size, Omega Engineering Inc. Stamford, CT) was inserted into one of the RBC samples, and temperature measurements were recorded by a Daq View USB Data acquisition system (Model OMB-DAQ 55, Omega Engineering, Inc. Stamford, CT) at 1 second intervals. Once the internal solution temperature reached -5 °C, ice formation was induced by touching the cryo-tubes with pre-chilled forceps. Samples were then held at -5 °C for two minutes for the dissipation of the latent heat of fusion. The protocol for further cooling depended on the cooling rate. Slow cooling involved using the methanol bath with a 1 °C/min rate setting to cool the samples to approximately -40 °C, then plunging the samples into liquid nitrogen (-196 °C) for 15 min. An intermediate, or medium cooling rate was achieved by immersing the samples into a dry ice – methanol slush (-78 °C) until the internal sample temperature reached approximately -40 °C, then plunging the samples into liquid nitrogen for 15 min. Finally, fast cooling involved immersion of sample tubes directly into liquid nitrogen for 15 min. The average cooling rate was calculated from temperature and time measurements as samples were cooled from -5 °C (after the dissipation of the latent heat of fusion) to -40 °C. Regardless of the cooling rate, RBC samples were thawed in a 37 °C circulating waterbath (Polyscience, VWR Scientific Products, Pennsylvania, USA) for approximately 15 min.

In addition to evaluating the effects of cooling rates and freezing solutions, the effect of liposome charge was evaluated by incubating RBCs with charged and uncharged liposomes, containing 0.3 M in their aqueous cores (4 mM lipid, in 0.3 M trehalose buffer). They were then frozen in HBS freezing solution using the fast cooling rate. To investigate whether liposome cryoprotective action is due to intracellular trehalose delivery or membrane phospholipid delivery, RBCs were incubated with negatively charged lipids or negatively charged liposomes containing either trehalose or saline (HBS) in their aqueous cores (4 mM lipid in 0.3 M trehalose buffer). After the incubation and density gradient separation, these RBCs were resuspended in 0.3 M trehalose solution and frozen to liquid nitrogen temperature using the fast cooling rate.

Post-thaw testing for all freezing experiments was performed immediately after thawing. RBCs maintained under the same conditions with respect to incubation conditions and density gradient separation, but without exposure to liposomes, were run in parallel for each freezing experiment and are referred to as control RBCs (c-RBCs, Table 6.1).

6.2.2. RBC Post-thaw Quality Assessment

Post-thaw percent hemolysis was determined for all freezing experiments by comparing the supernatant hemoglobin to total hemoglobin concentrations, using the cyanmethemoglobin Drabkin's method (Chapter 2, Section 2.2.1). Percent RBC post-thaw recovery was then calculated using the measured percent hemolysis values, according to the following equation:

$$\%\ RBC\ post\text{-}thaw\ recovery = 100 - \%\ hemolysis \qquad \text{(Eq. 6.1)}$$

Additional assessment of RBC membrane quality was performed on control RBCs and RBCs treated with negatively charged liposomes containing 0.3 M trehalose in their aqueous core, and were then frozen under the conditions that resulted in the highest RBC post-thaw recovery, according to results from the previous section. In this case, *in vitro* RBC quality was assessed pre-freeze (but post-liposome treatment) and post-thaw, using both the conventional RBC assays and more novel flow cytometry analysis, as described in Chapter 2. RBC morphology was assessed by staining the cells with a Hema 3 staining kit (Fisher Diagnostics, Middletown, VA) and examining the cells microscopically at 100x magnification, according to Chapter 2, Section 2.2.1. Flow cytometry was used to more closely examine the quality of RBC membrane by focusing on PS externalization and loss of CD47 Ag, using the protocol described in Chapter 2, Section 2.2.2.

Unless indicated otherwise, the data points presented in this chapter represent the mean values from six experiments with RBCs from three different donors, and error bars denote the standard deviation. The data were analyzed using commercial software (SigmaStat version 10.0, Systat Software, Germany and SPSS version 12.0, Lead Technologies, Charlotte, NC). Due to the small sample size (n=6 for each experimental condition), nonparametric statistical analysis was performed. Similarly to statistical analysis described in Chapter 2, the differences between the control and treated RBCs were examined using the Mann–Whitney U test, whereas the Kruskal-Wallis ANOVA was used to evaluate the effects of different conditions on RBC recovery. Probabilities less than 0.05 were considered significant.

6.3. Results

6.3.1. *The Effects of Cooling Rate and Freezing Solutions on l-RBC Response to Freezing*

Slow, medium and fast cooling rate profiles are shown in Figure 6.2. Cooling in a methanol bath resulted in a cooling rate of 0.95 ± 0.02 °C/min (Figure A); cooling in a dry ice – methanol slush resulted in a cooling rate of 73 ± 3 °C/min (Figure B); while plunging the samples into liquid nitrogen resulted in a cooling rate of 265 ± 12 °C/min (Figure B). Figure 6.3 illustrates the effects of different cooling rates and freezing solutions on the post-thaw recovery of c-RBCs and l-RBCs treated with negatively charged liposomes containing 0.3 M trehalose in their aqueous core. Liposome-treated RBCs showed significantly improved post-thaw recovery, regardless of cooling rate ($p<0.001$) and of freezing solution ($p=0.002$). However, while there was no statistically significant difference in the post-thaw recovery of l-RBCs between the medium and fast cooling rates in any of the freezing solutions (for NaCl $p=0.093$, for trehalose $p=0.700$ and for liposome freezing solution $p=0.394$), the recovery of l-RBCs was significantly lower in all three freezing solutions when the slow cooling rate was used (for NaCl p=0.032, for trehalose p=0.028 and for liposome freezing solution p=0.036). This effect was not consistently seen with c-RBCs. In addition to higher cooling rates, l-RBCs appeared to have better recovery in trehalose and liposome freezing solution, compared to NaCl. The recovery of l-RBCs frozen in the solution containing liposomes was significantly higher than that of l-RBCs frozen in NaCl solution for all three cooling rates (p=0.026 for slow cooling rate, p=0.009 for medium cooling rate and p=0.015 for fast cooling rate). However, there was no statistically significant difference in post-thaw recovery of l-RBCs frozen in liposome freezing solution and those frozen in trehalose solution (p=0.093 for slow cooling rate, p=0.394 for medium cooling rate and p=0.240 for fast cooling rate).

This analysis was also applied to c-RBCs. The post-thaw recovery of c-RBCs frozen in liposome freezing solution was significantly higher than that of l-RBCs frozen in NaCl solution for the medium and fast cooling rates (p=0.016 and p=0.029, respectively) but not for the slow cooling rate (p=0.700). Similar to l-RBCs, when the post-thaw recovery of c-RBCs frozen in trehalose was compared to those frozen in the liposome freezing solution, there was no statistically significant difference for all three cooling rates (p=0.400 for slow cooling rate, p=0.200 for medium cooling rate and p=0.548 for fast cooling rate).

6.3.2. The Effects of Liposome Treatment and Charge on RBC Response to Freezing

Figure 6.4 illustrates post-thaw recovery of c-RBCs and RBCs treated with charged and uncharged liposomes, both synthesized to contain 0.3 M trehalose in their aqueous core, then frozen in NaCl solution using a fast cooling rate (267 ± 14 °C/min). This figure confirms that RBC treatment with charged trehalose-containing liposomes improves RBC post-thaw recovery. The post-thaw recovery of charged l-RBCs was significantly higher compared to c-RBCs and to uncharged l-RBCs ($p<0.001$ for both groups). There was no statistically significant difference in post-thaw recovery between untreated c-RBCs and RBCs treated with uncharged liposomes ($p=0.073$).

6.3.3. The Effects of Freezing on l-RBC Membrane Quality

At the pre-freeze assessment, there was no apparent difference in morphology (Figure 6.5.A and Figure 6.6.A) or flow cytometry light scatter results (Figures 6.7-6.10) between c-RBCs and RBCs treated with negatively charged liposomes containing 0.3 M trehalose in their aqueous core. However, after these RBCs were frozen in 0.3 M trehalose solution, using a fast cooling rate, there were marked differences in the post-thaw quality between l-RBCs and c-RBCs. As illustrated in Figure 6.6.B, post-thaw c-RBCs show a larger degree of anisocytosis compared to post-thaw l-RBCs, shown in Figure 6.5.B. Flow cytometry analysis of post-thaw l-RBCs showed more events in the R1 gate with RBC-appropriate SSC versus FSC scatter (Figure 6.7 B) compared to post-thaw c-RBCs (Figure 6.8 B), which correlates well with percent hemolysis results. In addition, flow cytometry analysis of RBC PS externalization revealed that freezing considerably compromised membrane asymmetry of both l-RBCs and c-RBCs, as shown by the shift of RBC mean FL4 fluorescence intensity histogram to the right (Figure 6.9 A). However, when RBC mean fluorescence intensities were compared, the post-thaw PS expression of l-RBCs was significantly lower than that of c-RBCs (p=0.004, Figure 6.9.A). Flow cytometry data was analyzed by examining the percentage of RBC population that showed higher FL4 fluorescence due to PS exposure. These results show that in addition to lower recovery, post-thaw c-RBCs also had a significantly greater percentage of RBCs with exposed PS on their surface, compared to l-RBCs (p=0.010, Figure 6.9.B). Similar analysis was applied to examine RBC expression of CD47 antigen. Post-thaw l-RBCs expressed significantly more CD47 antigen on their membranes compared to c-RBCs (p<0.001, Figure 6.10.A), with a significantly lower percentage of RBCs with decreased CD47 expression (p=0.009, Figure 6.10.B).

6.3.4. On the Mechanism of Liposome Cryoprotection

Figure 6.9 investigates whether liposome cryoprotective action is due to intracellular trehalose delivery or membrane phospholipid delivery. This figure shows that the post-thaw recovery of l-RBCs was significantly higher compared to RBCs treated with negatively-charged lipids or c-RBCs (p=0.004 for both comparisons). Interestingly, there was no statistically significant difference in the post-thaw recovery between RBCs treated with liposomes containing trehalose in their aqueous core and RBCs treated with liposomes containing saline in their aqueous core (p=0.114). In addition, there was no statistically significant difference in recovery between c-RBCs and RBCs treated with negatively charged lipids which were not in the liposome form (p=0.243).

6.4. Discussion

Previous chapters of this thesis investigated the first half of the thesis hypothesis, and demonstrated that liposomal delivery can be used as a permeabilization strategy for the intracellular accumulation of trehalose with minimal injury to RBC membranes. By examining low temperature responses of liposome-treated RBCs, this chapter focuses on the second half of the thesis hypothesis. This chapter presents several interesting findings that may change how liposomes are perceived in the field of cryobiology.

The first significant finding of this chapter is that liposome treatment significantly improves recovery and membrane integrity of human RBCs following low temperature exposure. Figures 6.3 – 6.11 clearly demonstrate that liposomes modulate the RBC freezing response, resulting in cryoprotection. Although our results strongly support the hypothesis that liposomes behave as a novel CPA, the mechanisms underlying the liposome cryoprotective action is unclear. Figure 6.4 shows that charged trehalose-containing liposomes are more effective at protecting RBCs against cryoinjury than uncharged liposomes. Therefore, the second important finding of this chapter is that the extent of liposome-RBC interaction plays an essential role for cryoprotection. This finding is consistent with the results from the Chapter 5, which showed that charged liposomes interact more with RBCs and deliver more trehalose to the cell cytosol compared to uncharged liposomes. As protective properties of intracellular trehalsose have been previously described,[14; 15; 16; 17; 18; 19] it would be easy to presume that the extent of liposome-RBC interaction is important for the cryoprotection due to improved intracellular trehalose delivery. However, this hypothesis requires more rigorous investigation.

Therefore, RBCs were treated with liposomes containing either trehalose or saline in their aqueous core, and exposed to freezing conditions. The results of this experiment, shown in Figure 6.11, demonstrated that the post-thaw recovery of l-RBCs was significantly higher compared to c-RBCs, which reinforced the finding that the liposome treatment is cryoprotective. More interestingly, the results of this experiment clearly demonstrated that the liposome interaction with RBC membranes is responsible for the cryoprotection, rather than the delivery of intracellular trehalose. These results provide evidence that liposomes act as a novel CPA by modifying and/or preserving RBC membrane. Intracellular trehalose delivery is an added bonus of liposome treatment, but does not significantly improve cryoprotection. Many studies have reinforced the importance of achieving a threshold concentration of intracellular trehalose specific to each cellular system before a substantial improvement in cell recovery following low temperature exposure is apparent.[14; 17; 18; 48; 49; 50; 51; 52] It is not certain what intracellular trehalose concentration threshold is necessary for cryoprotection of human RBCs, but the results of this chapter clearly suggest that approximately 16 mM trehalose delivered by negatively charged liposomes (Chapter 5, Figure 5.6) is not sufficient. It may be necessary to further investigate approaches that enhance liposome intracellular trehalose delivery, as suggested in Chapter 5, in order to fully utilize the biostabilizing properties of trehalose in synergistic way with the membrane cryoprotective abilities of liposomes.

In addition, the results shown in Figure 6.11 provide another interesting finding. While RBCs trehalose- and saline-containing liposomes improved RBC post-thaw recovery, there was no cryoprotection when negatively charged lipids in the free and multilamellar form were incubated with RBCs. These results suggest that liposomes in a form of defined unilamellar vesicles are necessary to confer cryoprotection to RBCs. Furthermore, Figure 6.3 demonstrated that liposomes provided cryoprotection not only when incubated with RBCs prior to freezing, but also when present as a component of the extracellular freezing solution. Liposomes in the freezing solution provided a similar degree of RBC cryoprotection to extracellular trehalose (Figure 6.3). Together, these findings lead to several hypotheses on the mode of cryoprotective action of liposomes.

First, the liposome stabilizing property may be biomechanical. Liposome association with the RBC surface may result in the membrane "coating" effect. Liposomes tightly and loosely adsorbed to RBC membrane, hemi-fused liposomes, and free liposomes in solution may "coat" RBC membrane to provide a physical barrier that protects against cryoinjury due to solution effects during slow cooling. As described in Chapter 1, as ice forms in the extracellular solution, solutes become increasingly concentrated in the remaining unfrozen liquid, resulting in an osmotic pressure gradient and cryoinjury that is related to the "solution effects." Liposomes surrounding RBCs would become the first targets of this exposure to concentrated extra- and intracellular solutions at high sub-zero temperatures, leaving RBCs undamaged. The results of this chapter which demonstrate that liposome treatment improved post-thaw RBC morphology, decreased the extent of PS exposure and reduced the loss of CD47 antigen on RBC membranes (Figures 6.5-6.10) may be interpreted as the reduction in the "solution effects" cryoinjury. Although very unlikely at given experimental cooling rates, liposomes "coating" RBC membrane may potentially also protect against cryoinjury associated with progressive ice growth through membranes. The RBC membrane contains a large number of water channel proteins, or aquaporins (~3 x 10^5/ RBC), so the RBC membrane's permeability to water is very high.[53] As RBCs rapidly respond to changes in extracellular osmolality, they are less susceptible to the rapid-cool cryoinjury linked to intracellular ice formation, than to cryoinjury resulting from exposure to concentrated extra- and intracellular solutions at high sub-zero temperatures during slow cooling. Further studies are neede to determine whether liposome treatment affects aquaporin function and the permeability properties of the RBC membrane.

An extension of the biomechanical protection hypothesis is the "liposome repair hypothesis", first introduced by Leeuw *et al.*, who studied the ability of various agents, including liposomes, to protect against chilling injury of bovine sperm.[54] This hypothesis proposes that chilling injury results in the rearrangement of sperm membrane components, and exogenous liposome phospholipids might exert their protective effect, not by preventing these membrane alterations, but by repairing the local defects without changing the phospholipid content.[54] Kheirolomoom *et al.* further developed this hypothesis by demonstrating that the addition of liposomes to the lyophilization buffer substantially reduces hemolysis of freeze-dried human RBCs after rehydration.[20] Unlike nucleated cells, where membrane phospholipids participate in a state of dynamic equilibrium involving synthesis, transport and degradation, mature RBCs have no capacity to synthesize or actively regulate their phospholipid membrane composition.[20; 55; 56] Instead, RBCs depend on interactions with the components of plasma to maintain their membrane lipid composition.[20; 55; 56] As demonstrated by SEM images, the presence of liposomes in the extracellular milieu appears to prevent the formation of membrane blebs and protrusions during lyophilization.[20] Although these studies have examined cell injury during chilling and lyophilization, similar hypothesis can be applied to explain the results of this chapter, and propose that liposomes confer protection by repair of cryoinjury. Our results support this hypothesis by demonstrating that liposomes not only improve post-thaw RBC recovery, but also appear to exert their protective effect on post-thaw membrane quality. As illustrated in Figures 6.5-6.10, post-thaw liposome-treated RBCs show less anisocytosis and smaller degree of PS exposure and CD47 loss compared to control RBCs. Further studies are recommended to investigate the role of liposomes as cryoinjury repair vesicles.

In addition to biochemechanical hypotheses of liposome stabilizing actions, liposomes may protect RBCs from cryoinjury by biochemical means. Phospholipid and cholesterol delivery and/or exchange that occur(s) during liposome adhesion and fusion (as demonstrated in Chapter 4) may alter the RBC membrane composition, resulting in increased stability during freezing. A similar hypothesis was proposed by Parks *et al.*, who observed that phosphatidylcholine and lipoprotein vesicles protect bovine spermatozoa from chilling damage, and suggested that the modulation of membrane composition, specifically changes in the cholesterol / phospholipid ratio upon liposome treatment, is the likely mechanism.[57] This explanation may also be feasible for the cryoprotective action of liposomes. The fluorescence studies with hydrophobic R18 marker performed in Chapter 4 suggested that upon liposome-RBC interaction, there is a transfer of liposome phospholipids to RBC membrane with concomitant lipid mixing, resulting in lateral diffusion of liposome membrane phospholipids into the RBC lipid bilayer. This liposome-induced alteration in the RBC membrane composition may be cryoprotective.

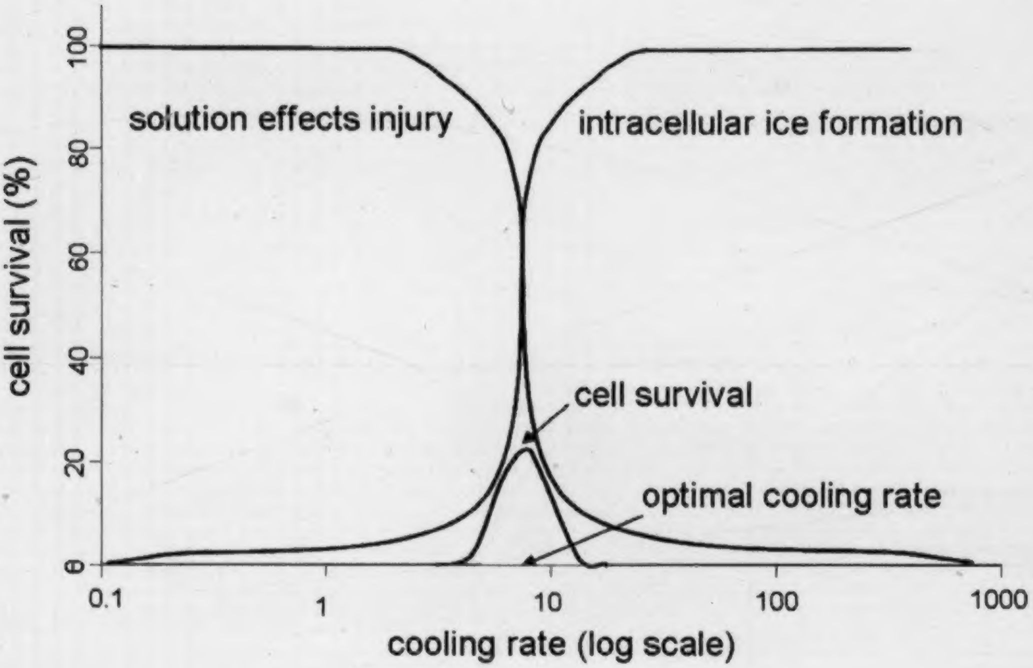
Additional hypotheses may be plausible. For example, Zeron *et al.* provided evidence that chilling injury of bovine spermatozoa and oocytes is related to the membrane T_m and the associated lateral phase phospholipid separation. [24] This group suggested that liposome association decreases cell membrane T_m, reducing damage associated with phospholipid aggregation and redistribution, as membranes pass through their T_m upon chilling.[24] As the role of membrane T_m in cryoinjury has not be as well defined as in chilling injury, and the human RBC membrane composition is quite different from bovine spermatozoa and oocytes, it is unclear whether the T_m hypothesis can be applied to explain cryoprotective actions of liposomes in RBC freezing. The investigations in this thesis did not address the changes in RBC membrane fluidity that may result from liposome adsorption and fusion. Further investigations are required to explore liposome-induced modification of RBC membrane T_m and the resulting effects on RBC response to freezing.

Figure 6.12 illustrates cell survival as a function of cooling rate of liposome-treated and control RBCs frozen in NaCl solution with slow, intermediate and fast different cooling rates, and compares our results to previously published survival curves of RBCs frozen without CPA,[27] RBCs frozen with a high concentration of permeating CPA glycerol,[31] and RBCs frozen in a nonpermeating sucrose CPA solution.[27] The recovery of the control RBCs frozen in NaCl solution is in agreement with previously published data.[27]. In addition, our results show that liposome cryoprotection is especially effective at higher cooling rates, which is consistent with optimal cooling rates RBCs when frozen without CPA or when frozen with a nonpermeating CPA. As plots of cell survival as a function of cooling rate usually exhibit an inverted U shape, increasing l-RBC recovery with the increase in cooling rates demonstrated in this chapter suggests that liposome treatment under presented experimental conditions result in recoveries that are on the left, or slow side of the inverted U survival curve (Figure 6.12). Therefore, the optimal cooling rate of liposome-treated RBCs is probably higher than approximately 265 °C/min. Cryoprotection conferred by liposomes appears to be more effective at lower cooling rates when compared to traditional nonpermeating CPA, such as sucrose (Figure 6.12). In contrast, the survival curve of RBCs frozen in high concentration of permeating cryoprotectant glycerol exhibits a much broader inverted U survival curve, resulting in higher RBC recovery at lower cooling rates compared to liposome-treated RBCs.[31] These findings suggest that liposomes may confer cryoprotection in a different way than traditional permeating and nonpermeating CPAs. Consequently, these finding may change how cryoprotection is viewed in the field of biopreservation, which may lead to research into a whole new class of CPAs. The positive outcomes of our freezing experiments suggest a great potential of liposome applications in the field of cryobiology. However, there are many scientific, clinical, technical and regulatory challenges before liposomes can be used as a CPA on a full-size RBC unit for transfusion purpose. Further research on applications of liposomes for RBC cryopreservation is warranted.

6.5. Conclusion

The results of this chapter demonstrate that liposomes significantly improve the recovery and membrane quality of human RBCs following cryopreservation. The beneficial effect of liposomes is likely related to modification and/or preservation of the RBC membrane, but additional studies

Figure 6.1. Cell survival as a function of cooling rate is illustrated, with the resulting injury occurring at slow cooling rates (solution effects injury) and at rapid cooling rates (intracellular ice formation).

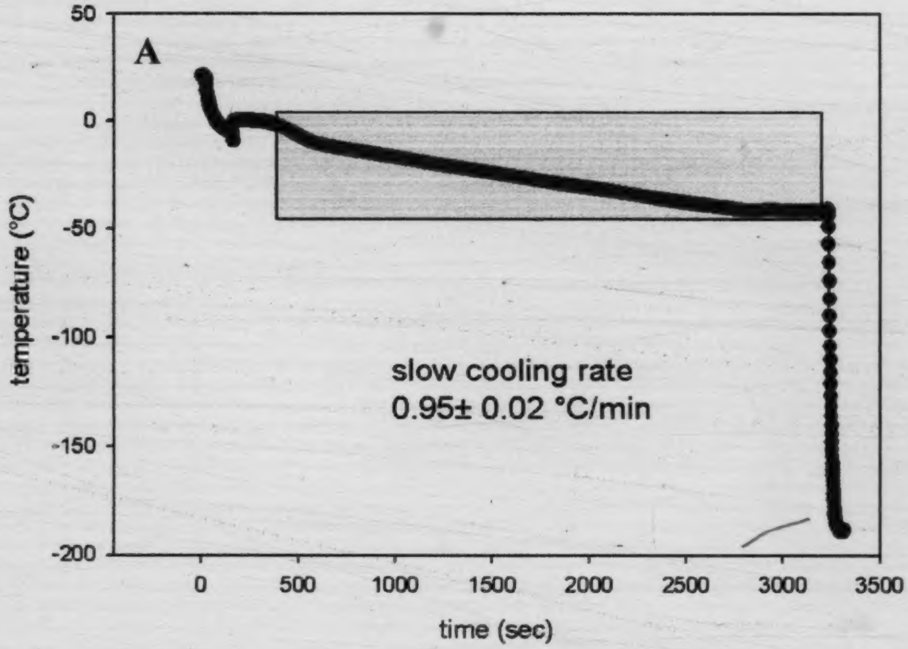


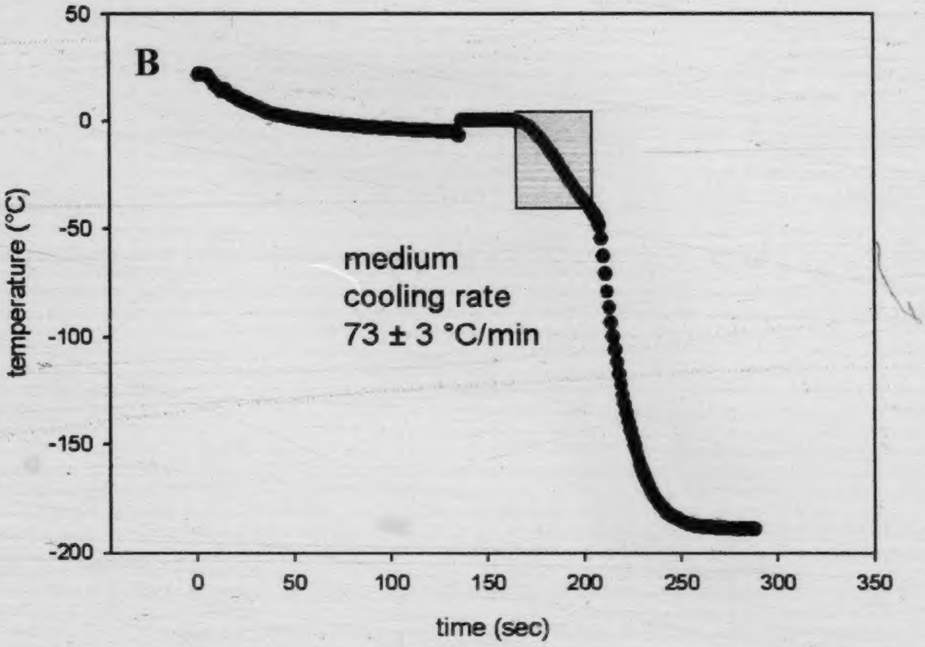
are clearly warranted to fully elucidate the mechanism of liposome cryoprotective action. As liposomes show significant promise as a novel biostabilizing agent, the research on this mechanism and the development of applications of liposomes in the biopreservation field will only continue to grow.

Table 6.1. Abbreviations used to describe different liposome preparations and treatments of RBCs.

Abbreviation	**Description**
trehalose liposomes	liposomes containing trehalose in their aqueous core
saline liposomes	liposomes containing HBS in their aqueous core (devoid of trehalose)
uncharged liposomes	liposomes with membrane consiting of DPPC:cholesterol (7:3 mol %)
charged liposomes	liposomes with membrane consiting of DPPC:PS:cholesterol (6:1:3 mol %)
l-RBC	liposome-treated RBCs
c-RBC	control RBCs, not treated by liposomes
lipid-RBC	RBCs treated with lipids which were not in the liposome form
(NaCl)	saline as a freezing solution
(treh)	0.3 M trehalose as a freezing solution
(lipo)	4 mM uncharged trehalose-liposomes in NaCl as a freezing solution

Figure 6.2. Cooling rate profiles: Figure A illustrates slow cooling rate, Figure B medium and Figure C fast cooling rate. The average cooling rate was calculated from temperature and time measurements as samples were cooled from -5 °C (after the dissipation of the latent heat of fusion) to -40 °C, as demonstrated by the blue box.




B
50
0
-50
-100
-150
-200
temperature (°C)
medium
cooling rate
73 ± 3 °C/min
0
50
100
150
200
250
300
350
time (sec)

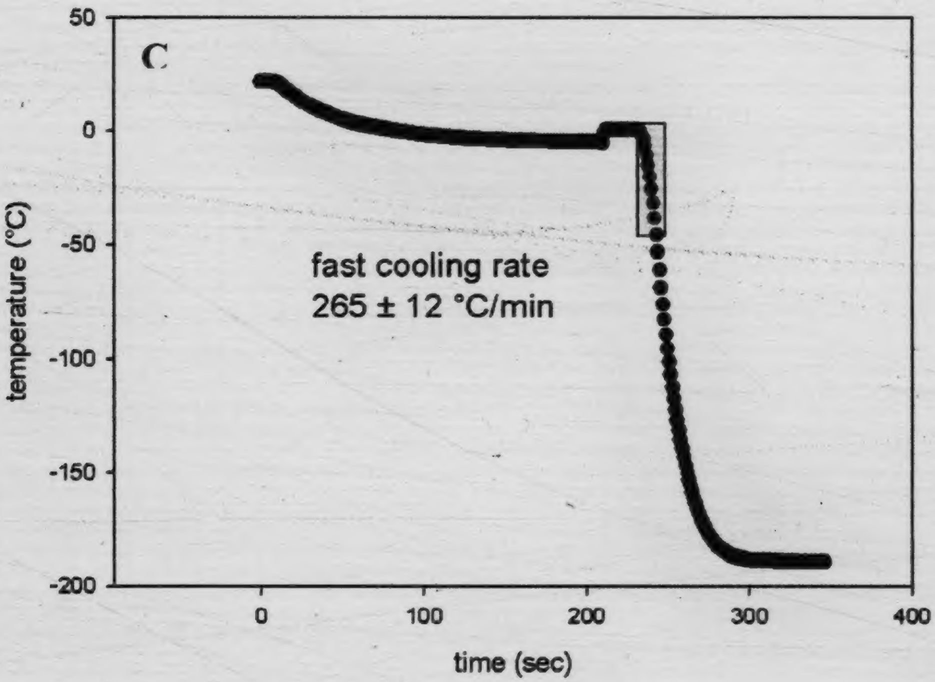

C
50
0
-50
-100
-150
-200
temperature (°C)
fast cooling rate
265 ± 12 °C/min
0
100
200
300
400
time (sec)

Figure 6.3. The effects of different cooling rates and freezing solutions on post-thaw recovery of liposome-treated and control RBCs. Please refer to Table 6.1 for the abbreviations used to describe different treatments of RBCs.

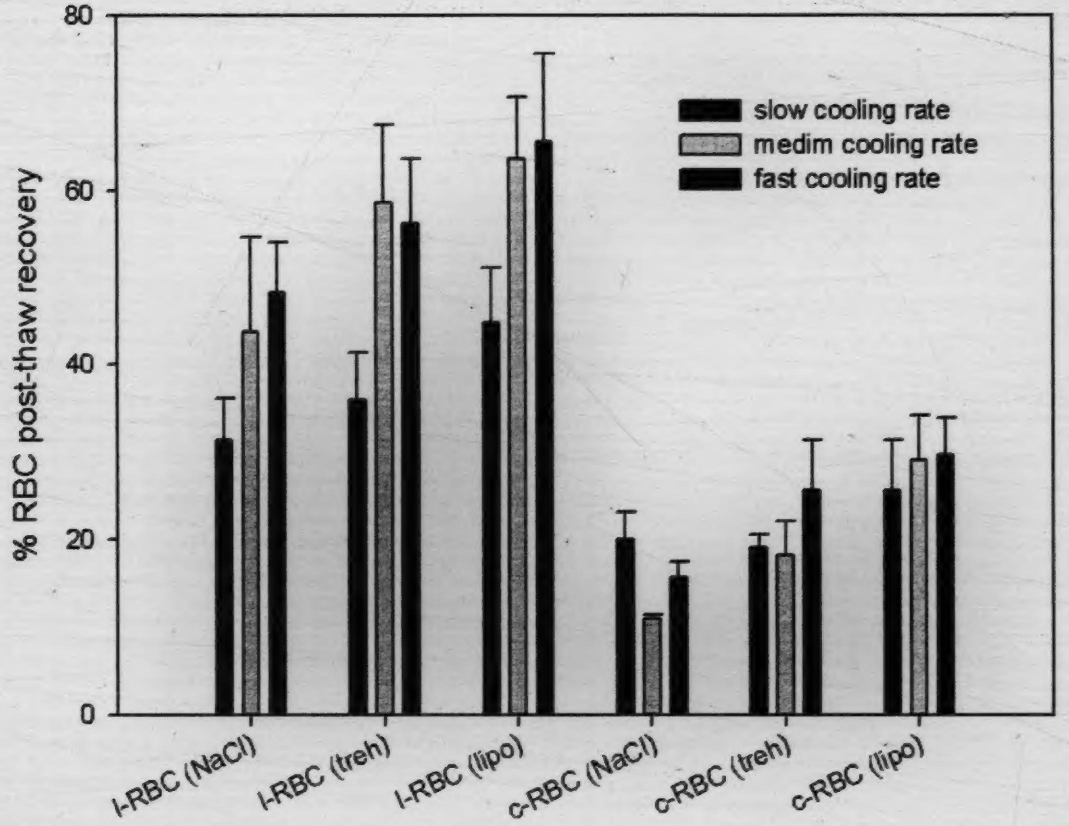


Figure 6.4. The effect of liposome charge on post-thaw recovery of liposome-treated and control RBCs. Both charged and uncharged liposomes were synthesized to contain 0.3 M trehalose in their aqueous core. RBCs were frozen in NaCl solution, using fast cooling rate (267 ± 14 °C/min). Please refer to Table 6.1 for the abbreviations used to describe different treatments of RBCs.

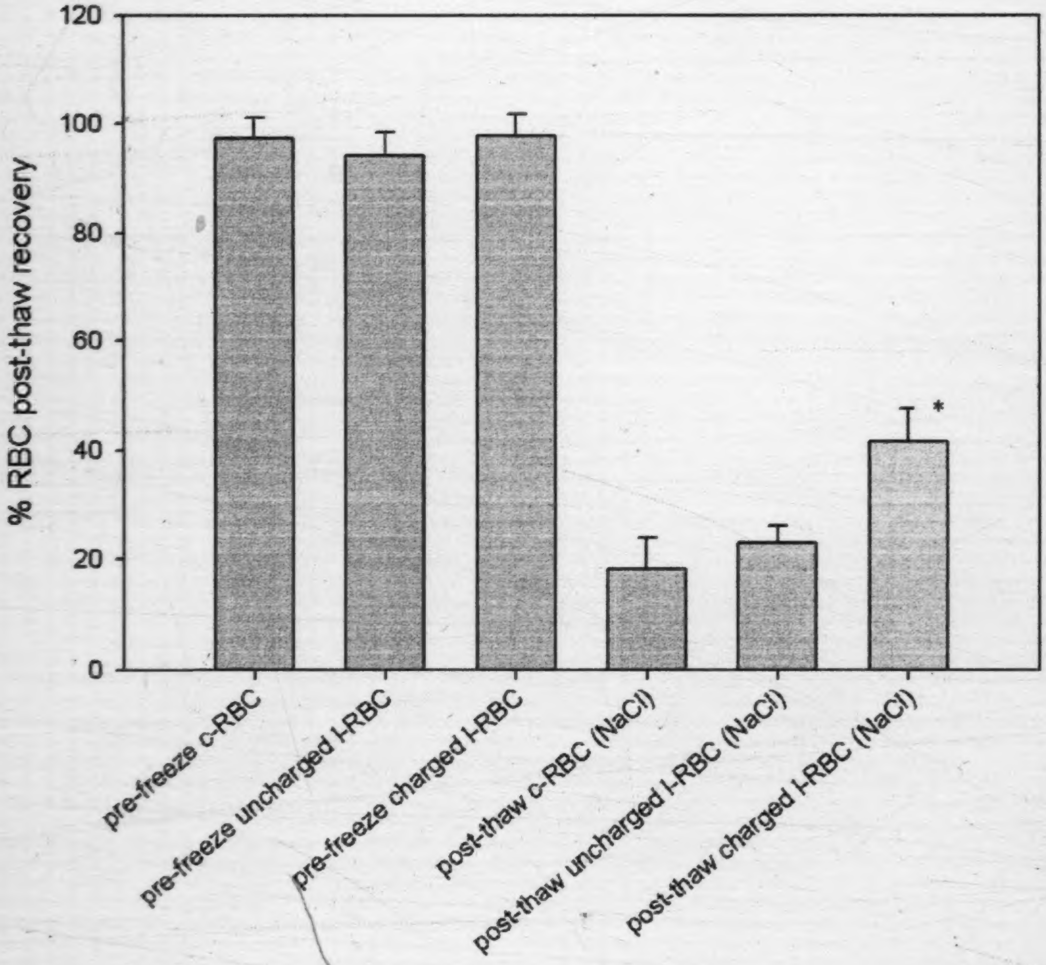


Figure 6.5. Pre-freeze (A) and post-thaw (B) morphology assessment of I-RBCs.

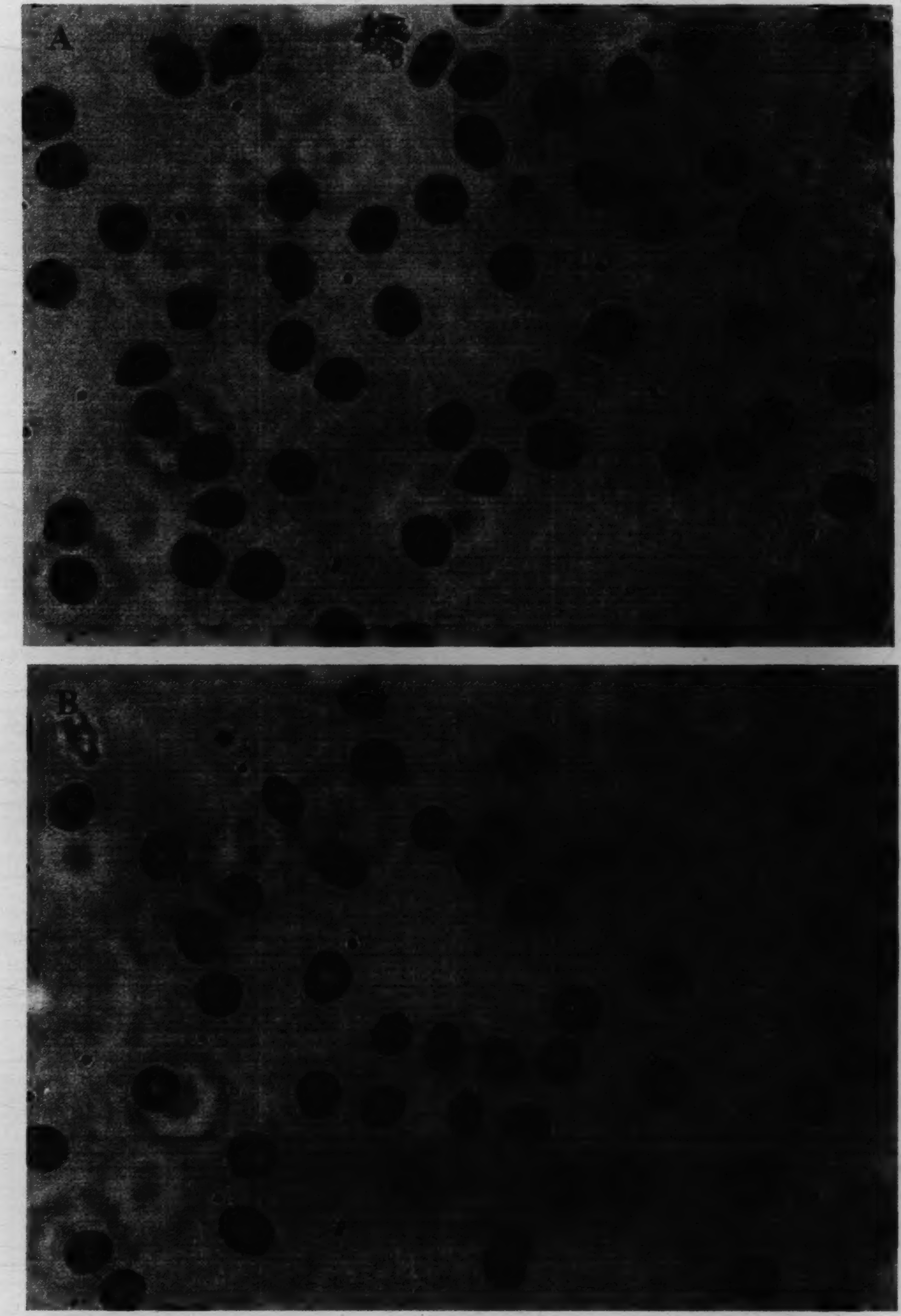


Figure 6.6. Pre-freeze (A) and post-thaw (B) morphology assessment of I-RBCs.

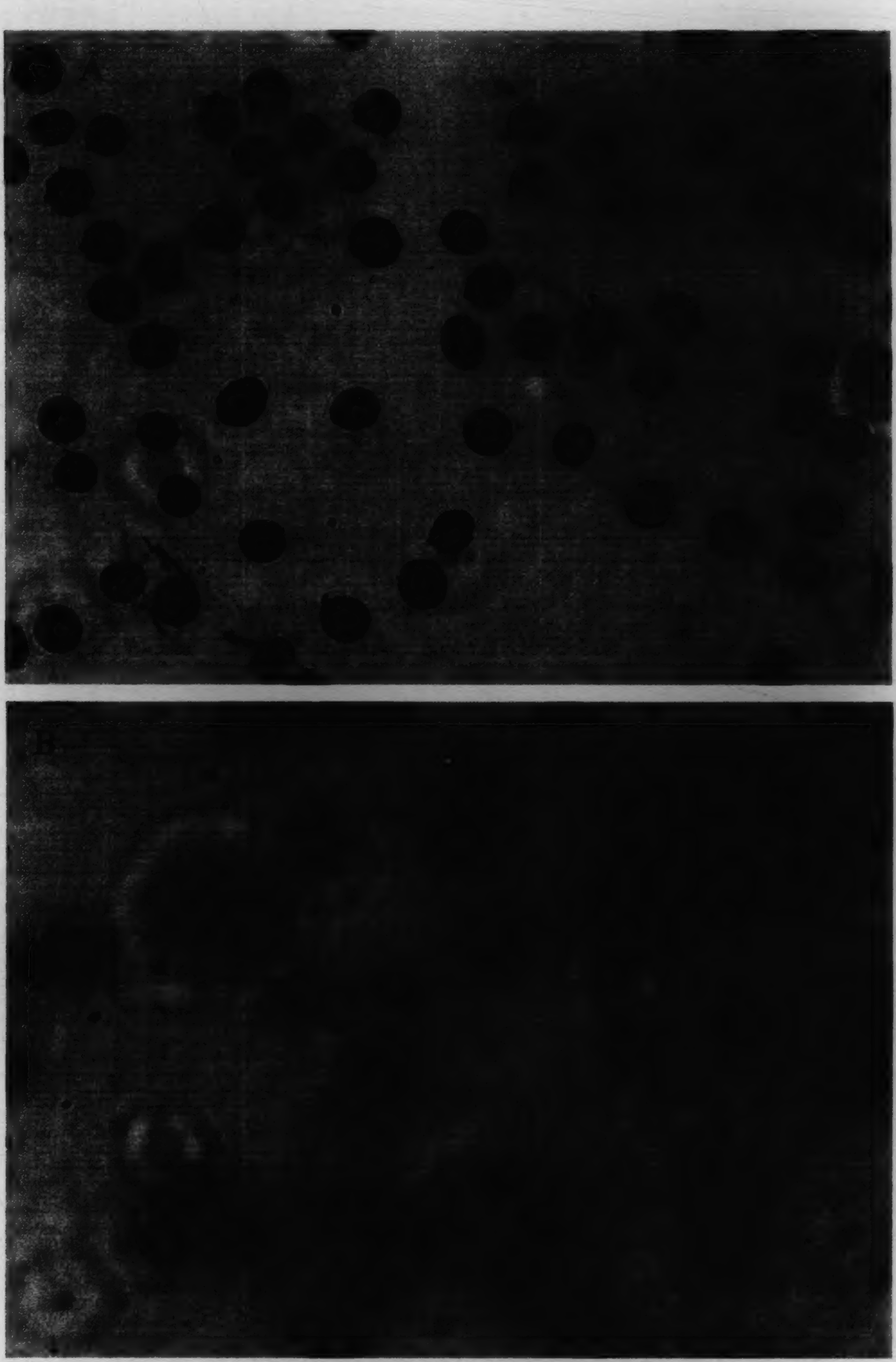


Figure 6.7. Flow cytometry analysis of liposome-treated RBCs, shown in the gate R1, pre-freeze (Figure A) and post-thaw (Figure B).

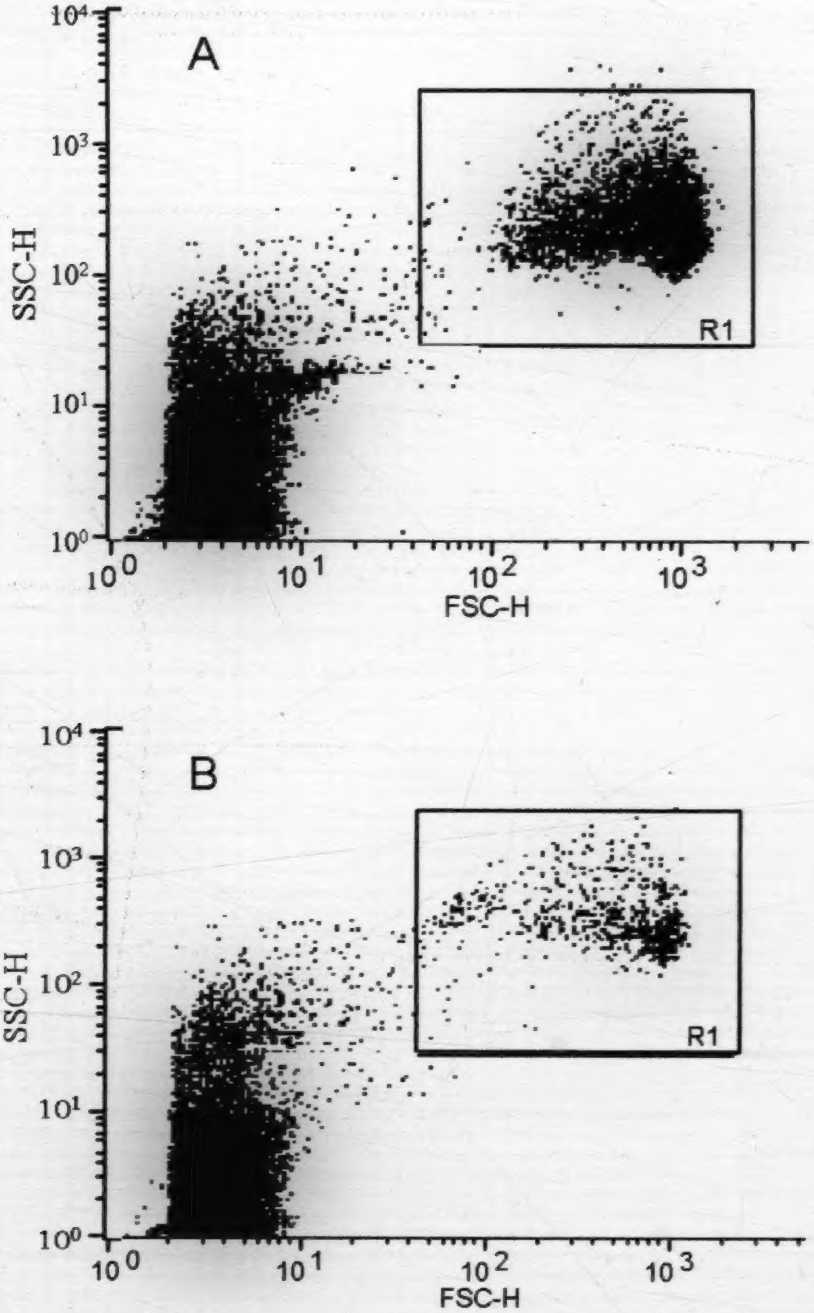


Figure 6.8. Flow cytometry analysis of control RBCs, shown in the gate R1, pre-freeze (Figure A) and post-thaw (Figure B).

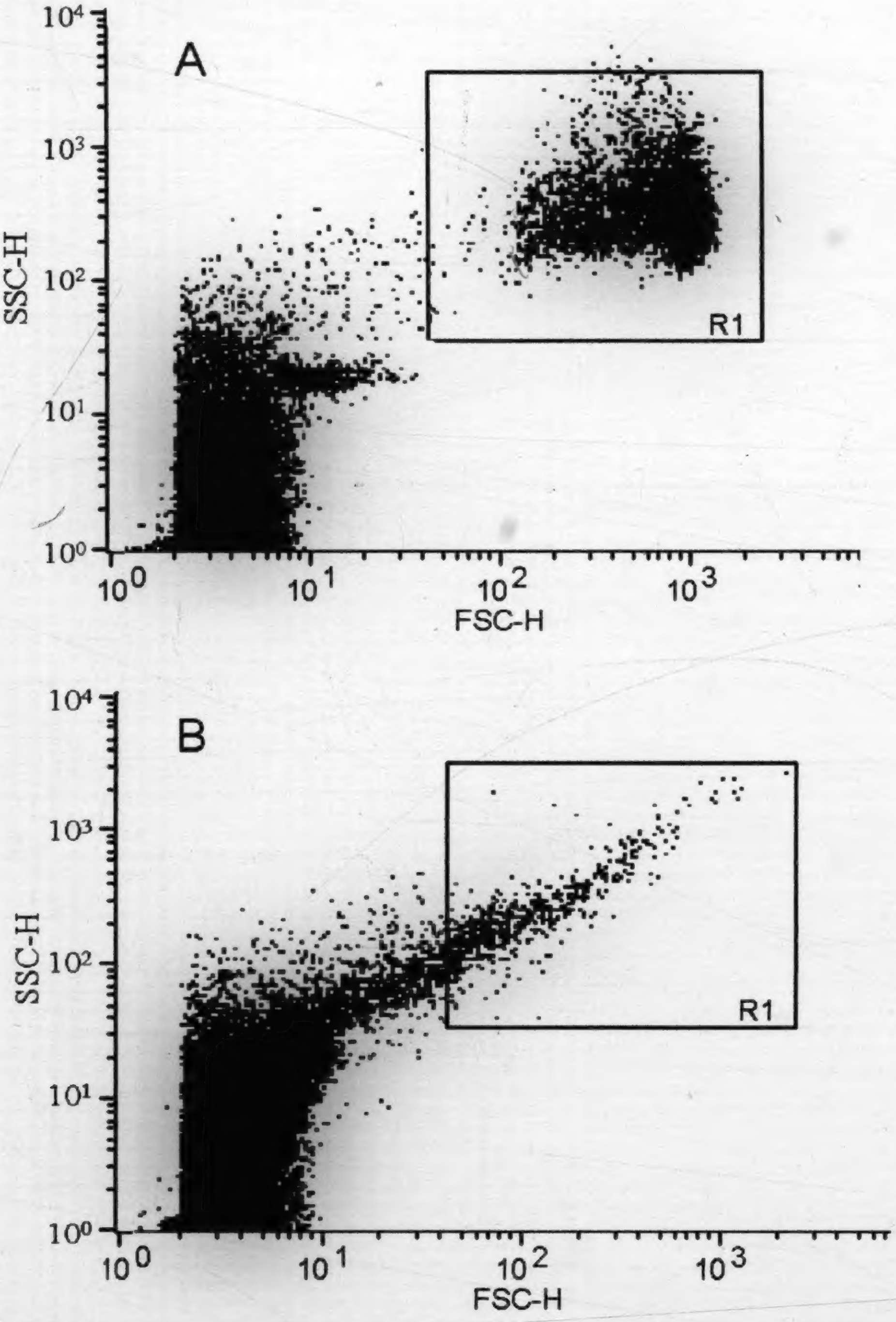


Figure 6.9. Pre-freeze and post-thaw flow cytometry analysis of PS exposure and CD47 expression of liposome-treated and control RBCs.

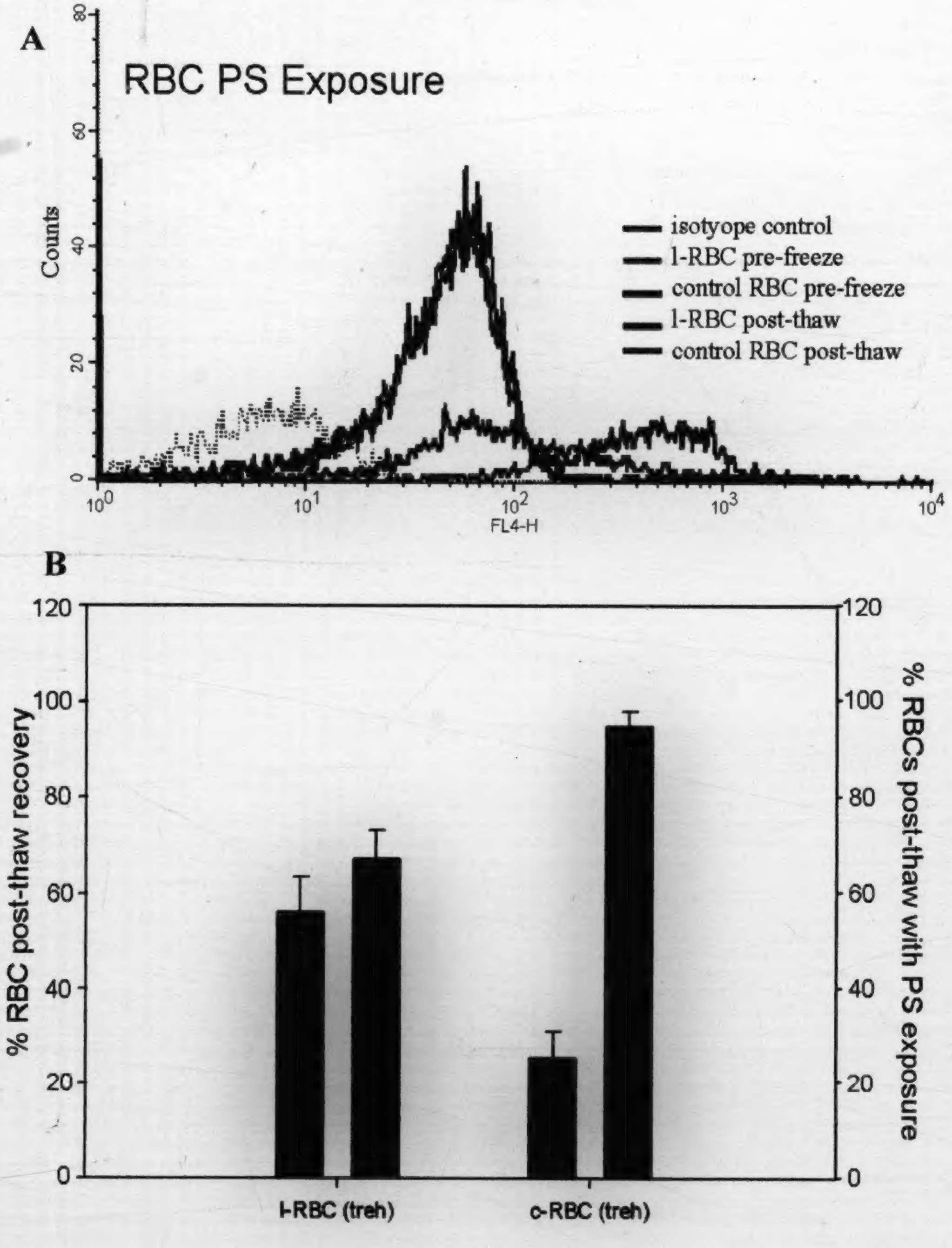


Figure 6.10. Pre-freeze and post-thaw flow cytometry analysis of PS exposure and CD47 expression of liposome-treated and control RBCs.

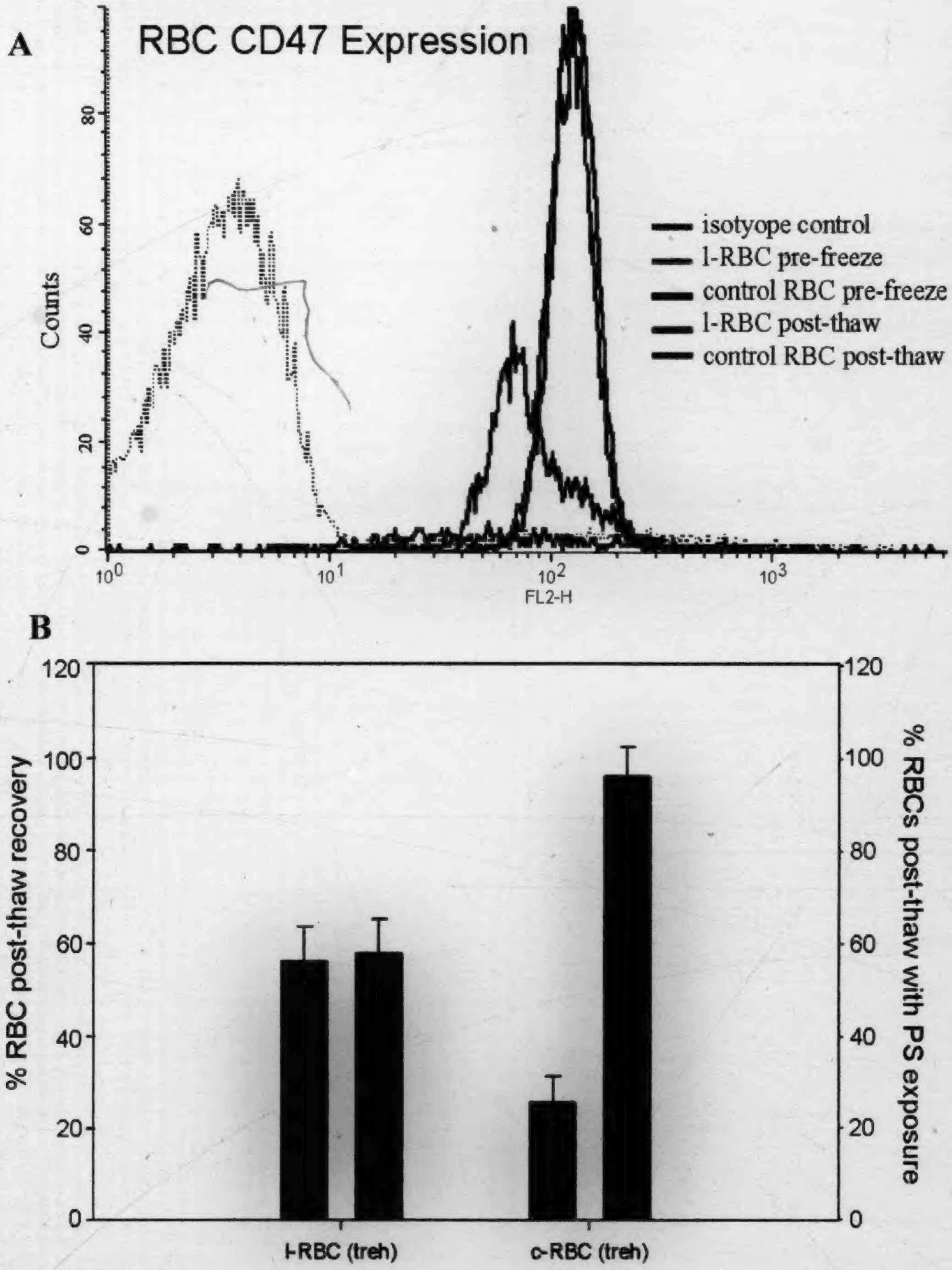


Figure 6.11. Phospholipid delivery during liposome-RBC membrane fusion plays an important role in improving RBC survival after freezing. Please refer to Table 6.1 for the abbreviations used to describe different treatments of RBCs.

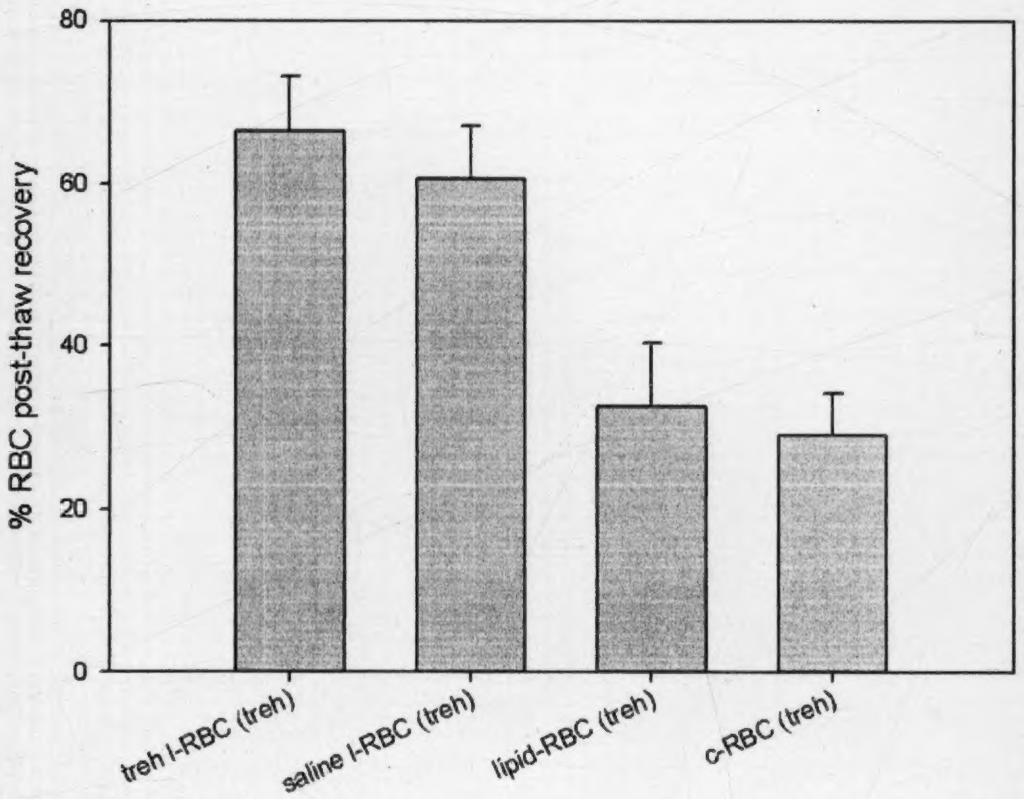
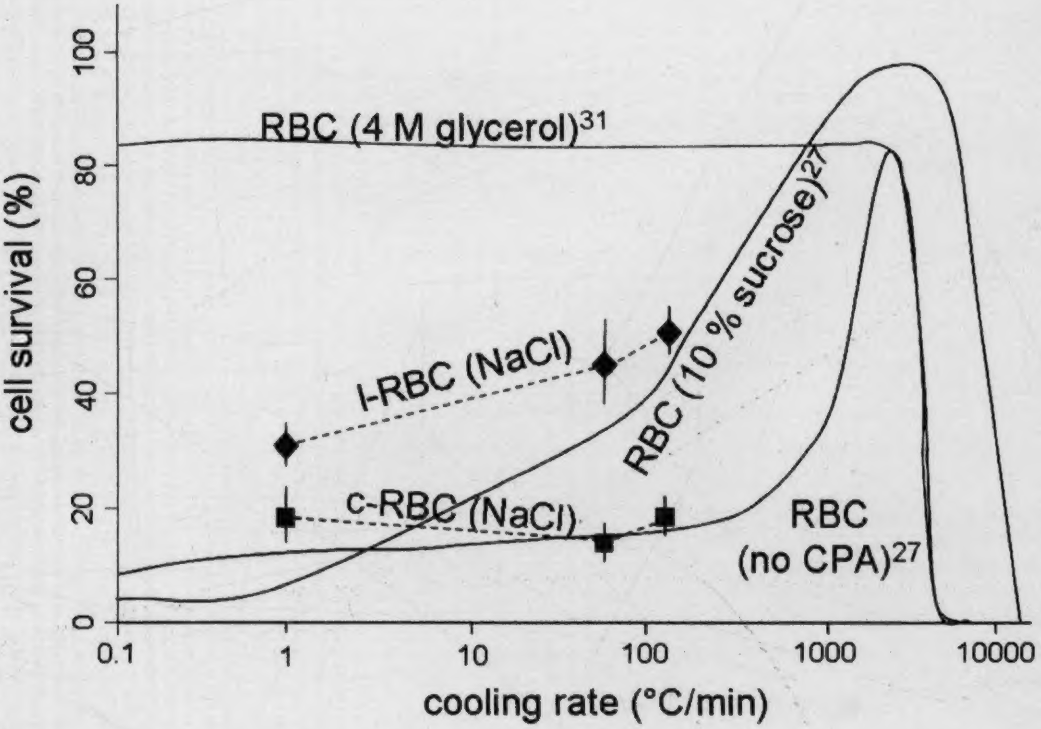


Figure 6.12. This graph illustrates cell survival as a function of cooling rate of liposome-treated (in green) and control RBCs (in black) frozen in NaCl solution with slow, intermediate and fast different cooling rates, and compares our results to previously published survival curves of RBCs frozen without CPA, (in black [27]) RBCs frozen with a high concentration of permeating CPA glycerol (in red [31]), and RBC frozen in a 10 % sucrose solution, a nonpermeating CPA (in blue[27]).



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Chapter 7

General Discussion and Conclusions

7.1. Review of Thesis Objectives

Cryopreservation is the process that maintains *ex vivo* biological structure and function by freezing and storage at ultra-low subzero temperatures. Cryopreservation of RBCs for transfusion is one of the most important applications of cryobiology research. The major advances in RBC cryopreservation occurred in the 1950s and 1960s, with the development of clinically-safe techniques for RBC freezing, storage, thawing and post-thaw processing. The current RBC cryopreservation is still based on these traditional methods using glycerol as a CPA, although they remain labour-intensive and expensive procedures that result in RBCs with shorter post-transfusion *in vivo* survival.[1; 2; 3; 4; 5; 6] The objective of this thesis has been to advance the field of RBC cryopreservation by expanding our understanding of the effects of traditional RBC cryopreservation on the RBC membrane quality, and by investigating a novel approach to RBC cryopreservation, involving trehalose and liposomes.

As pointed out by Meryman, learning from nature's own laboratory is the key to future advances in the field of cryobiology.[7] Examining the physiological and molecular adaptations of organisms able to withstand environmental stresses has recently lead to introduction of a whole new class of CPAs - nonpermeating intracellular CPAs, such as trehalose. Although trehalose posses many qualities that would make it an exceptional CPA, its full potential cannot be attained unless it is present on both sides of cell membrane.[8; 9; 10; 11; 12; 13] As mammalian plasma membranes are inherently impermeable to trehalose, the utility of trehalose as a CPA lies in introducing an innovative approach that will successfully load trehalose to cell cyotosol with minimal cell injury. That innovative approach was proposed by this thesis – liposomes. Since the early 1960s, the field of liposome research has been enormously expanding, with applications traversing numerous disciplines, including biochemistry, medicine, cell biology, food and cosmetic sciences, bioengineering, and pharmacology.[14] Only recently have liposomes started attracting attention in the biopreservation field. Therefore, this thesis incorporated liposome, cryobiology and transfusion medicine research to advance the science of RBC cryopreservation. Experimental results of five systematic studies have been presented in this thesis to test the hypothesis that liposomal delivery can be used as a permeabilization strategy for the intracellular accumulation of trehalose with minimal injury to RBC membranes, providing a novel approach for RBC cryopreservation.

The objective of the first experimental study of this thesis, described in Chapter 2, was to evaluate the quality of conventionally cryopreserved RBCs using novel indicators of RBC membrane lesion. The purpose of this study was to establish a baseline of RBC post-thaw quality when RBC are cryopreserved using a traditional high-glycerol method, before a new approach to RBC cryopreservation was to be further investigated. However, post-thaw RBC quality is a relative term. Although studies have shown that many *in vitro* parameters of deglycerolized RBCs are comparable to hypothermically stored RBCs, the 24 h *in vivo* survival of cryopreserved RBCs is significantly lower (while still meeting the minimum 75 % stipulated regulation).[1; 2; 3; 4; 5; 6] Changes in RBC surface expression of PS and CD47 and increased membrane microvesiculation have been recently documented features of the hypothermic storage lesion.[15; 16; 17; 18; 19; 20; 21; 22; 23] However, these markers have never been examined in deglycerolized RBCs. Therefore, this study evaluated the effects of glycerol-based RBC cryopreservation, 24 h post-deglycerolization hypothermic storage and pre-freeze storage length on these novel markers of RBC membrane injury, as well as correlated these measures to traditional RBC biochemical and biomechanical quality indicators. This study provided clinically important evidence that high-glycerol cryopreservation alone does not induce PS exposure, loss of CD47 expression and microvesiculation. However, prolonged pre-freeze storage was demonstrated to be a strong predictor of PS exposure or microvesiculation occurring after 24 h of post-thaw hypothermic storage. Since prolonged pre-freeze storage can affect RBC membrane asymmetry during the post-deglycerolization storage period, this study recommended that more defined criteria for this parameter should be adopted in clinical RBC cryopreservation. This study also recommended more research on the *in vitro* and *in vivo* effects of the novel markers of RBC membrane injury.

After establishing a baseline for traditionally cryopreserved RBCs and developing flow cytometry techniques to examine more subtle RBC membrane lesion, the next aim of this thesis was to develop a liposome-based method for the intracellular delivery of trehalose, as described in Chapter 3. This study described how existing techniques in the liposomal research can be adapted to synthesize and characterize trehalose-containing liposomes. Multiple methods were applied in this study to qualitatively and quantitatively assess synthesized trehalose-containing liposomes, including size, concentration, morphology, population homogeneity bilayer, membrane composition, and trehalose encapsulation. This chapter established trehalose-containing liposomes as a consistent and well characterized product, which is essential for controlled, reproducible and effective delivery of trehalose into mammalian cells for applications in cryopreservation.

The objective of the next study of this thesis, presented in Chapter 4, was to develop an understanding of the mechanism(s) by which trehalose-containing liposomes interact with human RBCs. Delineating the mechanism of liposomal interaction with RBCs is an essential step towards establishing the plausibility of using liposomes as tools for the intracellular delivery of trehalose. The results from this study, including fluorescence transfer and intracellular trehalose measurement, provided evidence to strongly support the hypothesis that liposomes deliver trehalose from their aqueous core to RBC cytosol. The presence of liposome-RBC membrane lipid mixing and content delivery suggested that the mechanism of interaction between unilamellar trehalose-containing liposomes and human RBCs involves adsorption of the vesicles to the RBC surface, as well as liposome content incorporation through membrane fusion. However, liposomes used in this study delivered only micromolar concentrations of intracellular trehalose.

As experimental parameters have been shown to play a significant role in liposome-cell interaction and post-interaction cell viability, the next study, presented in Chapter 5, investigated the effects of several experimental variables. These variables include the composition of liposome membrane and aqueous core, as well as the incubation conditions, such as liposome to cell ratio, incubation time, temperature, and medium on liposome-cell interaction, trehalose loading and RBC membrane quality. This study provided valuable experimental evidence for identifying conditions which enhance liposome interaction with RBCs, such as negative charge of liposome membrane, prolonged incubation time, and higher incubation temperature. The results of this study also demonstrated that liposome treatment did not cause significant adverse effects on RBC membrane quality, which is critical if liposomes are to be used as a membrane permeabilization technique. RBC membrane damage was shown to be more dependent on the incubation time and temperature, rather than on liposome composition and concentration. Therefore, this study suggested that improving liposome trehalose delivery involves balancing the efficiency of trehalose uptake with the minimization of cell membrane injury resulting from prolonged liposome-cell incubation at higher temperatures.

The final study of this thesis, described in Chapter 6, investigated the effects of trehalose-containing liposomes on RBC response to freezing. The post-thaw RBC *in vitro* quality was evaluated using both conventional assays, such as percent hemolysis, and novel flow cytometry assays, including PS exposure and the expression of CD47, which were established in Chapter 2. The results of this study provided evidence to several important findings. First, liposome treatment was found to significantly improve recovery and membrane quality of human RBCs following cryopreservation. The extent of liposome-RBC interaction was shown to play an essential role for cryoprotection, as charged trehalose-containing liposomes were more effective at protecting RBCs against cryoinjury than uncharged liposomes. This study also demonstrated that different extracellular solutions and cooling rates play an important role in liposome cryoprotective effect. Liposome-treated RBCs showed an improved post-thaw survival if higher cooling rates were applied and extracellular freezing solutions contained trehalose or liposomes. Furthermore, this study provided important evidence that the liposome cryoprotective effect is not due to the delivery of intracellular trehalose, but is likely related to modification and/or preservation of the RBC membrane. In addition, this study proposed several hypotheses on the mechanism of liposome cryoprotection for future investigations.

7.2. Contributions to Science and Future Directions

This thesis has made a number of unique contributions that will advance the scientific knowledge in the fields of cryobiology and transfusion medicine. The work presented herein has provided a further understanding of RBC membrane injury that occurs during traditional clinical RBC cryopreservation. In view of the potential detrimental immunomodulatory, procoagulatory and microcirculatory effects of the described markers of RBC membrane injury, the clinical implications of our findings warrant further study. Furthermore, this thesis describes the first account of using liposomes as membrane permeabilization strategy for intracellular delivery of trehalose. This permeabilization strategy may help circumvent the key impediment to using many different nonpermeating intracellular CPAs – inherent impermeability of the mammalian cell membrane to large molecular weight and/or charged molecules. In the process of investigating liposomes as intracellular trehalose delivery vesicles, this thesis has contributed comprehensive analysis of interactions of trehalose-containing liposomes and human RBCs, including modes of interactions and the effects of various experimental parameters on liposome-RBC interaction, trehalose loading and RBC membrane quality. Finally, this thesis has advanced the knowledge base of cryobiology by providing novel evidence that liposomes can be used to exert a cryoprotective effect on human RBCs, which may change how cryoprotection is viewed, leading the research into a whole new class of CPAs. The findings of this thesis have the potential to guide a new approach to RBC cryopreservation that might alleviate problems associated with the traditional glycerol-based cryopreservation methods used in transfusion medicine. Ultimately, this knowledge will contribute to the design of novel preservation systems, not only for RBCs, but also for other clinically and biologically significant cells.

I believe that major achievements in cryobiology lie ahead. The fundamental biophysical principles of freezing and cryoinjury pioneered in the 1960s have set a solid stage for incremental and empirical advances over the last 50 years. I believe that future progress of cryobiology science will involve learning from Mother Nature and applying the traditional biophysical approach to understanding the mechanisms of stress tolerance in natural organisms. For the field of cryobiology to advance, there is a crucial need for new collaborations of divergent ideas, disciplines, technologies and applications. Melding a multidisciplinary platform of cryobiology knowledge through cooperation and scientific discussion will be a key factor in evolving ideas on how to harness the natural ability of organisms to survive freezing and desiccation. This thesis provides cryobiology researchers new techniques and avenues of thought to explore. My hope is that it will inspire the next generation of scientist to further pursue multidisciplinary ideas that will make major advances in the field of cryobiology.

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